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**Plant biomass-acting enzymes produced by the  
ascomycete fungi *Penicillium subrubescens* and  
*Aspergillus niger* and their potential in biotechnological  
applications**

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Cover: *Penicillium subrubescens* FBCC1632 on minimal medium amended with (upper row left to right) apple pectin, inulin, wheat bran, sugar beet pulp, (lower row left to right) citrus pulp, soybean hulls, cotton seed pulp or alfalfa meal (photos: Ronald de Vries).

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Dedicated to my sweetheart wife and daughter

## Abstract

Plant biomass contains complex polysaccharides that can be divided into structural and storage polysaccharides. Plant biomass is a major carbon source utilized by chemoheterotrophic microorganisms, such as filamentous fungi to grow and produce energy. Plant biomass-degrading fungi secrete a wide range of extracellular enzymes to convert complex plant biomass into metabolizable compounds. As a result, they are able to colonize and degrade a wide range of organic materials. The industrially and biotechnologically important fungal genera *Penicillium* and *Aspergillus* are among the best-studied decomposers of plant biomass polysaccharides.

The focus of this study was on two ascomycete fungi, *Penicillium subrubescens* (FBCC 1632) and *Aspergillus niger* N402 (ATCC 64947), and their potential for producing extracellular enzymes for the decomposition of plant biomass polysaccharides. Here, a new species, native Finnish isolate, *P. subrubescens* sp. nov. was described. It belongs to the section *Lanata-Divaricata* subgenus *Aspergilloides*. *P. subrubescens* was selected amongst 200 fungal strains screened for their ability to produce polyfructan inulin-degrading enzymes. Inulin is a storage polysaccharide located in the roots and tubers of flowering plants. *P. subrubescens* was identified based on the combined analysis of phenotype together with molecular phylogenetic analysis and extrolite data. *P. subrubescens* produced inulinase more efficiently than it did invertase. Only fructose, not fructo-oligosaccharides was detected as the endproduct of inulin hydrolysis indicating exo-type inulinase activity.

The ability of *P. subrubescens* and *A. niger* to produce plant structural polysaccharide-degrading enzymes was studied by growth profiling and in plant biomass-containing liquid cultures. These data indicated similar (hemi-)cellulolytic capacities for these fungi. The extracellular enzyme mixtures of *P. subrubescens* and *A. niger* were used in the hydrolysis of wheat bran, sugar beet pulp and a mixture of these. Its favourable ability to hydrolyse complex plant-derived biomasses indicated that *P. subrubescens* has the potential to produce biotechnologically important enzyme mixtures.

A new feruloyl esterase, FaeC of *A. niger* was cloned and heterologously produced from the plant biomass-acting enzymes. The biochemical properties of recombinant FaeC (rFaeC) were characterized, and the hydrolysis of wheat arabinoxylan and sugar beet pectin by rFaeC released both ferulic and *p*-coumaric acid. The synergistic activity of rFaeC and xylanase was detected in hydrolysis of plant biomass-derived substrates. The induction of *faeA*, *faeB* and *faeC* gene expression in the presence of various phenolic compounds and complex polysaccharides was examined. The differing expression levels of the three *fae* genes suggests that the corresponding enzymes can act cooperatively, leading to improvement in the efficiency of plant biomass decomposition.

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## List of original publications

This thesis is based on the following publications:

- I Mansouri S, Houbraeken J, Samson RA, Frisvad JC, Christensen M, Tuthill DE, Koutaniemi S, Hatakka A, Lankinen P. 2013. *Penicillium subrubescens*, a new species efficiently producing inulinase. *Antonie Van Leeuwenhoek* 103:1343-57.
- II Mäkelä MR\*, Mansouri S\*, Wiebenga A, Rytioja J, de Vries RP, Hildén K. 2016. *Penicillium subrubescens* is a promising alternative for *Aspergillus niger* in enzymatic plant biomass saccharification. *New Biotechnology* 33: 834–841.  
\*Equal contribution.
- III Dilokpimol A, Mäkelä MR, Mansouri S, Belova O, Waterstraat M, Bunzel M, de Vries RP, Hildén KS. 2017. Expanding the feruloyl esterase gene family of *Aspergillus niger* by characterization of a feruloyl esterase, FaeC. *New Biotechnology* 37: 200–209.

The publications are referred to in the text by Roman numerals.

## Contribution of the author to the publications

- I Sadegh Mansouri participated in the design of the study, performed most of the cultivations and conducted the experimental work, participated in the data analysis, and wrote the article together with the co-authors.
- II Sadegh Mansouri participated in the design of the study, performed the fungal cultivations and extractions, the experimental work on enzyme activity and enzymatic hydrolysis, participated in the data analysis, and wrote the article together with the co-authors.
- III Sadegh Mansouri participated in the design of the study, performed the fungal cultivations and extractions, the experimental work on gene extraction and cloning, participated in the data analysis, and wrote the article together with the co-authors.



## Abbreviations

AA	Auxiliary activity	CAZy	Carbohydrate active enzyme database
ABF	Arabinofuranosidase	CAZyme	Carbohydrate active enzyme
ABN	Endo-arabinase	CBH	Cellobiohydrolase
ABTS	2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	CBM	Carbohydrate binding module
ABX	Exo-arabinase	CDH	Cellobiose dehydrogenase
AFC	$\alpha$ -Fucosidase	CE	Carbohydrate esterase
AG	Apiogalacturonan	CREA	Creatine agar
AGD	$\alpha$ -1,4-Glucosidase	CYA	Czapek yeast autolysate agar
AGL	$\alpha$ -Galactosidase	CYAS	CYA supplemented with 5% NaCl
AMY	$\alpha$ -Amylase	DG18	Dichloran 18% glycerol agar
Ara	Arabinose	DP	Degree of polymerization
ATCC	American Type Culture Collection	EGL	Endo-glucanase
AUG	$\alpha$ -Glucuronidase	FA	Ferulic acid
AX	Arabinoxylan	FAE	Feruloyl esterase
AXE	Acetyl (xylan) esterase	FBCC	Fungal Biotechnology Culture Collection
AXH	Arabinoxylan $\alpha$ -arabi-nofuranohydrolase	Frc	Fructose
AXL	$\alpha$ -Xylosidase		
BGL	$\beta$ -Glucosidase		
BXL	$\beta$ -Xylosidase		
CaM	Calmodulin		

GAL	$\beta$ -Endogalactanase	MEA	Malt extract agar
Gal	Galactose	MFA	Methyl ferulate
GalA	Galacturonic acid	MND	$\beta$ -1,4-Mannosidase
		MnP	Manganese peroxidase
GH	Glycoside hydrolase	MpCA	Methyl <i>p</i> -coumarate
Glc	Glucose	MSA	Methyl sinapinate
GRAS	Generally recognized as safe	NFCC	National Fungal Culture Collection
GT	Glycosyl transferase	PAE	Pectin acetyl esterase
HFS	High fructose syrup	PE	Pectin esterase
HG	Homogalacturonan	PEL	Pectin lyase
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection	PG	Polygalacturonase
		PGA	Endo-polygalacturonase
INU	Endo-inulinase	PGL	Polygalacturonate lyase
INX	Exo-inulinase	PGX	Exo-polygalacturonase
ITS	Internal transcribed spacer	PL	Polysaccharide lyase
		PLY	Pectate lyase
LAC	$\beta$ -1,4-Galactosidase	PME	Pectin methyl esterase
LiP	Lignin peroxidase		
LPMO	Lytic polysaccharide monooxygenase	PMG	Polymethyl-galacturonase
MAN	$\beta$ -1,4-Mannanase	PMGL	Polymethyl-galacturonate lyase
Man	Mannose		
MCA	Methyl caffeate	RG	Rhamnogalacturonan

RGAE	Rhamnogalacturonan acetyl esterase	WB	Wheat bran
RGL	Rhamnogalacturonan lyase	XEG	Xyloglucan-active $\beta$ - 1,4-endoglucanase
RHG	Endo-rhamno- galacturonase	XGA	Xylogalacturonan
Rha	Rhamnose	XGH	Endo- xylogalacturonase
SBP	Sugar beet pulp	XGX	Exo- xylogalacturonase
SF	Subfamily	XLN	$\beta$ -1,4-Endoxylanase
SUC	Invertase	Xyl	Xylose
TLC	Thin layer chromatography	YES	Yeast extract sucrose agar
VP	Versatile peroxidase		

# 1. Introduction

Plant biomass polysaccharides-hydrolysing enzymes have long been used in the food, feed, textile, brewing and baking, pulp and paper, and pharmaceutical industries. These enzymes originate mainly from microorganisms, such as filamentous fungi. The microbial enzymes are also used for hydrolysis of plant biomass into platform sugars (de Vries et al., 2002; Penttilä et al., 2003). Plant biomass is the most abundant renewable resource for the production of biofuels and chemicals (Mäkelä et al., 2015). In the environment, 200 billion tonnes of plant biomass are produced annually, and its decomposition relies mainly on wood-decaying and litter-decomposing fungi (Hatakka & Hammel, 2010).

Plant biomass polysaccharides are mainly comprised of structural and storage polysaccharides (Berg et al., 2007). Plant structural polysaccharides originate from wood and offshoots of the forest, agricultural, and food processing industries, in addition to aquatic plants and algae (Demirbas, 2001). They partake in the formation of the structural framework of the cell walls in plants lignocellulosic biomass. The structure of the plant lignocellulosic biomass is a rigid, complex with a tightly organized matrix of intricate polysaccharides and the aromatic polymer lignin (Gilbert, 2010). Structural polysaccharides have versatile applications in the food, pulp and paper, fibre and clothing, cosmetic and pharmaceutical industries, in addition to the production of biofuels and value-added byproducts (May, 1990; Zheng et al., 2009; Shokri & Adibkia, 2013).

Plant storage polysaccharides such as starch and inulin are mainly homopolymers of glucose (Glc) or fructose (Frc) respectively. They are

considered as reserve carbohydrates stored in the plant storage organs. When needed, storage polysaccharides are hydrolysed and the monomeric sugars released are accessible to the living cells for the production of energy and biosynthesis (Berg et al., 2007). Starch, which is a cheap organic substance, has a wide variety of applications in the food and pharmaceutical industries (Burrell, 2003). It is used as a thickener, water binder, and emulsion stabilizer, in addition to stiffening and gelling agent. Starch is used as a gluing agent in nonfood sectors such as the paper, textile, and fertilizer industries (Copeland et al., 2009).

Inulin, which is certified as generally recognized as safe (GRAS) by the FDA (U.S. Food and Drug Administration), has been used as a carbohydrate source to produce high fructose syrup (HFS). It has also been used as a sweetener and for fat replacement (Closa-Monasterolo et al., 2013). In addition, oligofructans have recently been recognized as renewable, inexpensive and abundant raw materials for the production of biofuel, citric acid, single-cell protein, and other biochemicals (Nakamura et al., 1996; Zhao et al., 2010; Chi et al., 2011).

To release energy from plant biomass, diverse technologies such as direct combustion, chemical, thermochemical and biochemical processes are used (Cherubini, 2010). Biochemical conversion involves the combined activity of various carbohydrate active enzymes (CAZymes) listed in the CAZy database, (<http://www.cazy.org>) for depolymerization of the polysaccharides into fermentable monosaccharides (Yang et al., 2011). The monomeric sugars released can be further fermented into biofuels and other value-added products.

Filamentous fungi are among the most efficient degraders of plant biomass, producing oxidative and hydrolytic enzymes leading to efficient carbon recycling in the environment (Kubicek et al., 2009; Mäkelä et al., 2014). Cellulose, hemicellulose, and pectin, which are the major plant cell wall polysaccharides, are decomposed by fungal species belonging to the phyla Basidiomycota and Ascomycota, while the complete degradation of the aromatic compound lignin is mainly caused by white-rot basidiomycetes (Lundell et al., 2010). *Aspergillus niger*, *Trichoderma reesei*, and *Penicillium* species are the most well-studied ascomycete filamentous fungi for the production of CAZymes (Brown et al., 1987; Chaabouni et al., 1994; de Vries et al., 2002; Jørgensen et al., 2005; Maeda et al., 2013; Marjamaa et al., 2013). Penicillia are generally saprobes and soil-dwelling fungi, which play important roles in natural carbon recycling. They have the ability to grow on a broad range of organic substances such as dead plant materials, and they are able to degrade a wide variety of plant-originated biomass by their lignocellulose-degrading enzymes (Adsul et al., 2007; Li et al., 2007; Terrasan et al., 2010).

## **1.1 Plant biomass polymers and compounds**

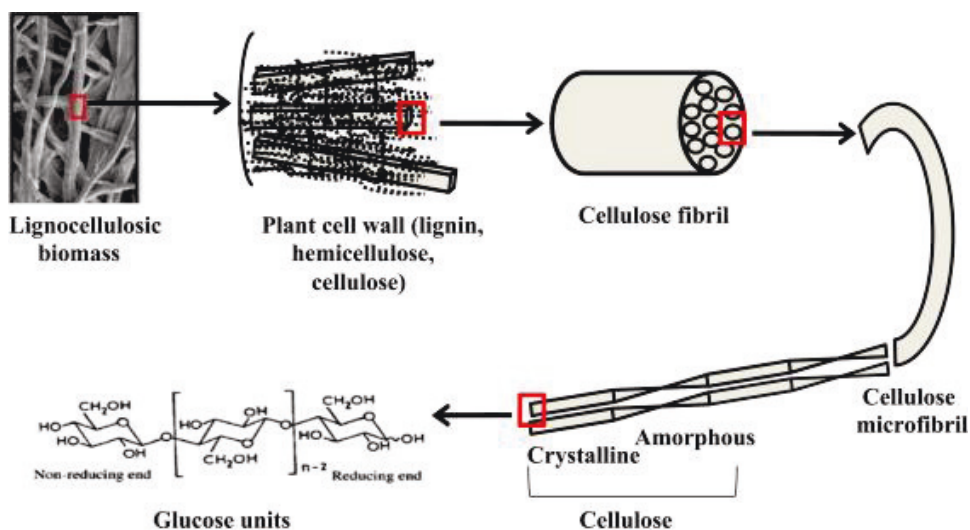
Plant biomass typically contains plant cell wall polysaccharides, i.e. cellulose and hemicelluloses, and lignin. In addition, the storage organs, e.g. seeds, tubers and roots, contain reserve carbohydrates, starch and inulin.

### **1.1.1. Structural polymers and compounds**

#### **1.1.1.1. Cellulose**

Cellulose is the most abundant polysaccharide in plant cell walls constituting 40-45% of their dry weight (Sjöström, 1993). It is a homopolymer of Glc

consisting of a linear chain of hundreds to over 10 000 of  $\beta$  (1 $\rightarrow$ 4)-linked D-Glc units (Hatakka & Hammel, 2010) (Fig. 1). Its average degree of polymerization (DP) varies from 6000 to 14 000 (Harris & Stone, 2008). Cellulose is the major constituent of both primary and secondary plant cell walls and is organized into high-tensile-strength microfibrils (Alberts et al., 2002).

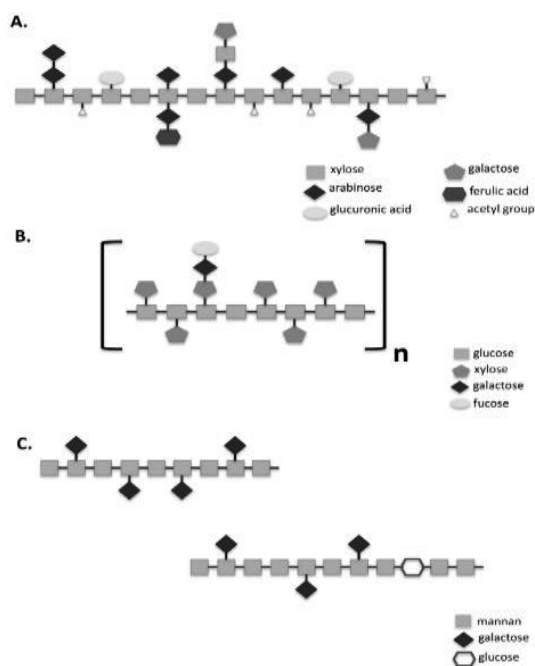


**Figure 1.** Illustrative scheme of cellulose in plant cell walls. Reprinted from Kuhad et al. (2016) with permission from Elsevier.

Cellulose microfibrils are held together by intra- and intermolecular hydrogen bonds and are arranged in parallel orientation to form macrofibrils. Macrofibrils are highly ordered, recalcitrant and resistant to hydrolysis and form highly insoluble crystalline and partially amorphous structures of cellulose (Guerriero et al., 2010).

### 1.1.1.2. Hemicelluloses

Hemicelluloses constitute 20-30% of plant dry matter (Sjöström, 1993). They form a mixed and complex polymeric network of pentoses and hexoses, and their detailed structure and redundancy vary widely depending on the plant species and cell-wall structures (Scheller & Ulvskov, 2010). Together with lignin, hemicellulose contributes to cell-wall rigidity and strength by interacting with the network of cellulose fibres. Hemicelluloses are usually classified according to the main monosaccharide units present in the backbone of the polymer. Xylan, xyloglucans, and mannan are the main types of hemicellulose structures (van den Brink & de Vries, 2011) (Fig. 2).



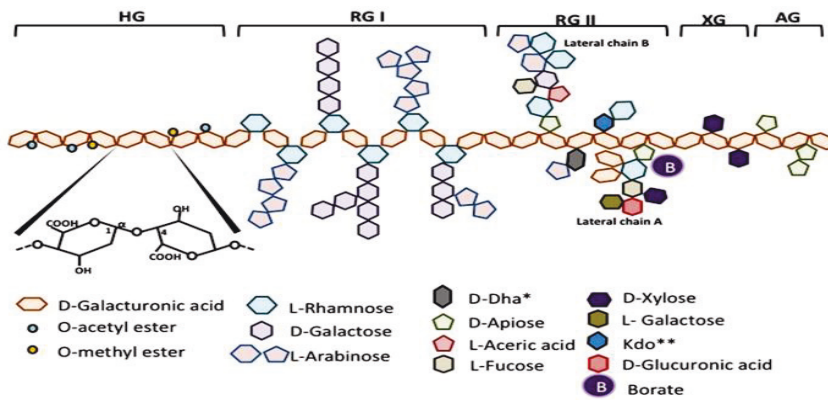
**Figure 2.** Schematic representation of the three major hemicellulose structures. A) Xylan, B) Xyloglucan, C) Galactomannan (upper left) and Galactoglucomannan (lower right). Reprinted from de Souza (2013) under the Creative Commons Attribution License (CC-BY 3,0).



Xylan is the main hemicellulose in the cell walls of cereals and hardwoods. Its backbone is built of  $\beta$ -1,4-linked D-xylose (D-Xyl) repeating units. Xyloglucan, which is the most abundant hemicellulose in the primary cell walls of dicots, possesses a backbone of  $\beta$ -1,4-linked D-Glc repeating units (Fry, 1989). Mannan (galactomannan or galactoglucomannan) is the main hemicellulose fraction in softwood, and it is also found in hardwood cell walls (Moreira & Filho, 2008). Its backbone consists of  $\beta$ -1,4-linked D-mannose (D-Man) repeating units, which can be interrupted by  $\beta$ -1,4-linked D-Glc (Srivastava & Kapoor, 2005). Mannan can be either galactomannan or galactoglucomannan, depending on the backbone interruption or substitution. The backbone of xylan, xyloglucan and mannan can be substituted by D-galactose (D-Gal), D-Xyl, L-arabinose (L-Ara), L-fucose, D-glucuronic acid (D-GluA), acetate groups and hydroxycinnamic acids such as ferulic, and *p*-coumaric acids (van den Brink & de Vries, 2011). Compared with cellulose, hemicellulose is branched and has a lower DP of about 100-200 sugar residues (Sjöström, 1993) and, therefore, it is more soluble and susceptible to biodegradation.

#### **1.1.1.3. Pectin**

Pectic substances are a group of plant cell wall-associated polymers containing D-galacturonic acid (D-GalA) residues that are covalently linked by  $\alpha$ -1,4-linkages (Mohnen, 2008). They are mainly found in the primary cell walls and middle lamellae of terrestrial and some aquatic plants where they participate in binding cells together by forming additional cross-linkages between cellulose and hemicellulose (Caffall & Mohnen, 2009; Rytioja et al., 2014) (Fig. 3).



**Figure 3.** Schematic depiction of pectin structure. HG; homogalacturonan, RG; rhamno-galacturonan, XG; xylogalacturonan, AG; apiogalacturonan. Reprinted from Leclerc et al. (2009) under the Creative Commons Attribution License (CC-BY 3,0).

Homogalacturonan (HG) is the simplest and most abundant pectic polysaccharide. HG, which accounts for 65% of pectin structure, is an  $\alpha$ -1,4-covalently linked linear homopolymer of GalA (Tan et al., 2013). It can be methyl-esterified at the C-6 carboxyl group and acetylated at the O-2 or O-3 positions, leading to the formation of the smooth region (SR) of pectin (Sjöström, 1993; Aro et al., 2005). There are also four other structurally different and more complex classes of HG-substituted heteropolymers, namely rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), xylogalacturonan (XGA), and apiogalacturonan (AG) (Ridley et al., 2001).

RG-I is the most complex pectic polysaccharides and accounts for 20-30% of pectin structure. It contains repeating disaccharide units of  $\alpha$ -1,4-D-GalA and  $\alpha$ -1,2-L-rhamnose ( $\alpha$ -1,2-L-Rha), and it shows wide variation in the type and number of sugars such as D-Gal and L-Ara attached to the L-Rha residues (Willats et al., 2001). RG-II is built of seven to nine  $\alpha$ -1,4-linked GalA units consisting of four side chains (A-D), that are decorated by 12 different glycosyl residues (O'Neill et al., 2004; Caffall & Mohnen, 2009). RG-II is usually dimerized in plant cell walls by which the HG domains are cross-linked to form

pectin macromolecule networks (Matsunaga et al., 2004). RG-II is structurally conserved among plant species, indicating its important role in cell-wall functioning (O'Neill et al., 2004).

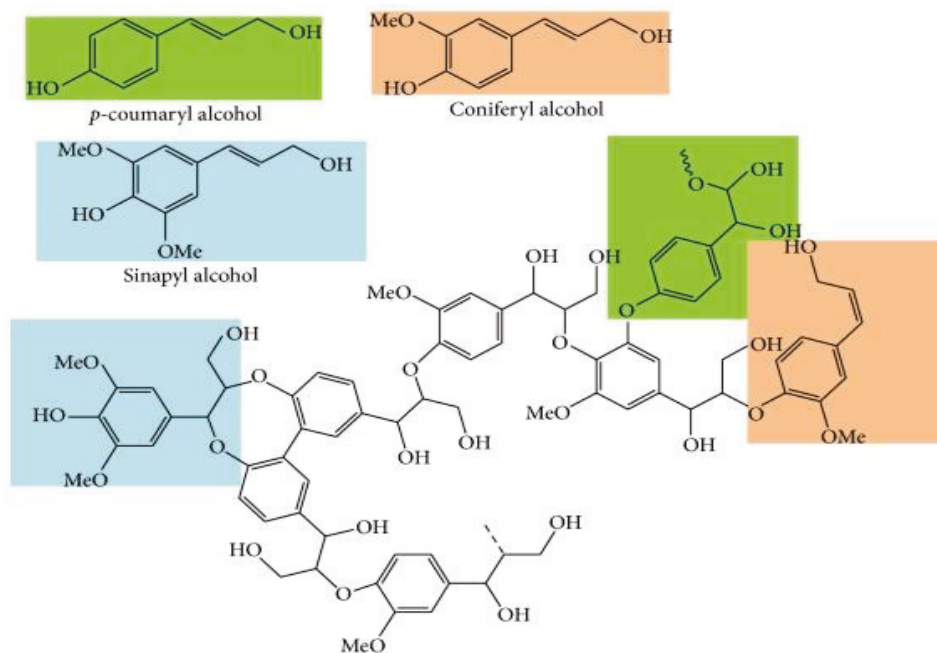
Attachments of mono- and oligosaccharides to the RG backbone lead to the formation of the hairy region (HR) of pectin (Pérez et al., 2000; Ridley et al., 2001). RG-I and RG-II are covalently linked to the HG backbone to cross-link the xyloglucan (Popper & Fry, 2008). XGA is a substituted HG that has a  $\beta$ -linked D-Xyl at the C-3 position (de Vries & Visser, 2001). AG is a HG with 3-linked D-apiose, which is mostly abundant in aquatic and marine plants (Caffall & Mohnen, 2009).

#### **1.1.1.4. Lignin**

Lignin is a complex cross-linked phenolic polymer with an amorphous, high-molecular-weight structure (Fig. 4), forming an extensive network within the plant cell wall (Hatakka & Hammel, 2010). It is commonly present in the secondary cell walls and middle lamellae of various plant species (Müsel et al., 1997). Lignin is situated between the cellulose, hemicellulose and pectin in the cell wall, forming covalent linkages to hemicellulose and building up lignin-carbohydrate complexes (Chabannes et al., 2001). It contributes to the vital physiological and structural roles of the cell-wall by (i) providing mechanical support and strength to the plant structure, (ii) hindering biological degradation by digestive enzymes produced by pathogenic microorganisms (Martone et al., 2009) and (iii) reducing cell-wall permeability, leading to efficient transfer of water and nutrients throughout the stems of vascular plants (Sarkanen & Ludwig, 1971).

Lignin is formed from phenylpropane units, e.g. *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units that are covalently linked together to form a net-like structure (Mäkelä et al., 2015). Phenylpropane units are formed via radical-mediated polymerization of three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols (Boerjan & Ralph, 2003).

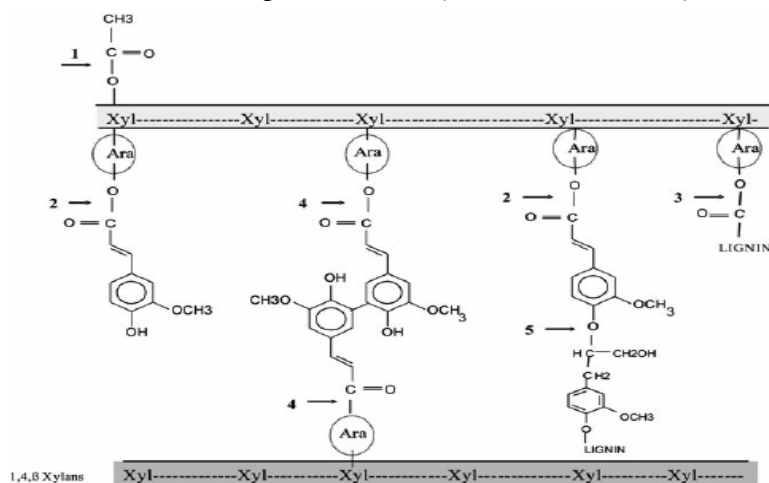
The proportions of the lignin structural units vary widely depending on the plant type and origin. Softwood lignin consists of G-units together with trace amounts of H- and S-units (Dence & Lin, 1992), whereas hardwood lignin is composed mostly of G- and S-units. H-units are most common in the lignin of grasses and flowering plants (Adler, 1977; Lewis & Yamamoto, 1990).



**Figure 4.** Schematic representation of lignin structure. Reprinted from Lee et al. (2014) under the Creative Commons Attribution License (CC-BY 3,0).

### 1.1.1.5. Hydroxycinnamic acids

Hydroxycinnamic acid derivatives such as ferulic, *p*-coumaric, sinapinic, and caffeic acids, which are phenylpropanoids or aromatic acids, are found in plant cell wall structures (Benoit et al., 2008). They cross-link the structural polymers of the cell walls (Fig. 5), leading to cell-wall rigidity and strength (Ishii, 1997; Carnachan & Harris, 2000). Their molecular structures are comprised of both carboxyl and hydroxyl groups indicating a bifunctional quality through which they are able to form both ether and ester linkages (de O. Buanafina, 2009). Oxidative coupling of hydroxycinnamates, which can be catalysed by hydrogen peroxide and peroxidases, leads to the formation of new dehydrodimers of ferulate (Ralph et al., 1994; Fry, 2004) or photoinduced cyclodimers of ferulate and *p*-coumarate (Turner et al., 1993).



**Figure 5.** Simplified structure of ferulic acid (FA) cross-linking arinoxylan (AX) in grass cell walls. The  $\beta$ -1,4-linked xylan backbone is represented by dotted lines and the side sugar, arabinose (Ara), are shown in circles. Ester and ether bonds are shown by arrows as follows: (1) acetyl group, (2) ester linked FA to AX, (3) arabinose-lignin, (4) 5–5 –ester linked FA dimer cross-linking AX chains, (5) FA ether linked to lignin. Reprinted from de O. Buanafina (2009) with permission from Elsevier.

Ferulates, diferulates, and *p*-coumarate are recognized as important structural components of the cell walls of gramineous and graminoid plant species (Kroon et al., 1999). The main polysaccharide matrix of the primary cell walls in graminoids is glucuronoarabinoxylan (Scheller & Ulvskov, 2010; Bartley et al., 2013), which is composed of a  $\beta$ -1,4-linked Xyl backbone with arabinofuranose substitution at the O-3 position (Bartley et al., 2013). The glucuronoarabinoxylan of cell walls in graminoid plant species is esterified with ferulic and *p*-coumaric acids (Ishii, 1997; de O. Buanaфина, 2009) by O-5 ester linkages between the carboxyl groups of phenolic compounds and the hydroxyl groups of sugars, mainly  $\alpha$ -L-Ara (de Vries & Visser, 2001). Ferulic acid (FA) is also able to mediate the lignin and polysaccharide connection by ether linkages formed by the hydroxyl group of ferulate and a phenolic lignin monomer (de Vries & Visser, 2001; Sun et al., 2002; de O. Buanaфина, 2009).

It is assumed that the main biological roles of diferulates are to strengthen the primary and secondary cell walls resulting in (i) hindering of biomass degradation by fungal enzymes, (ii) prolonging or stopping cell-wall degradation by normal ruminant digestive tract flora and (iii) limiting cell-wall growth (Kroon et al., 1999; Santiago et al., 2007, 2008; Benoit et al., 2008; Lanoue et al., 2010).

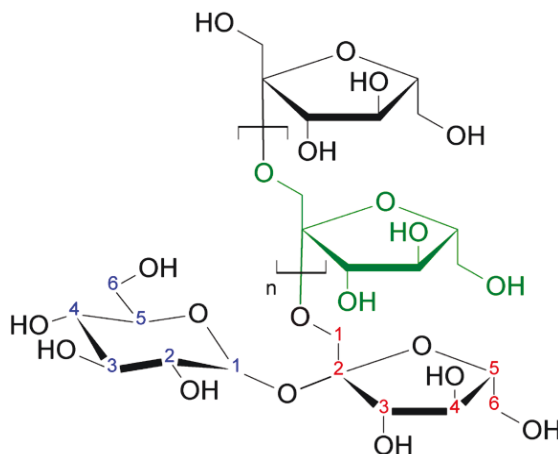
### **1.1.2. Storage polymers**

#### **1.1.2.1. Inulin**

Inulin is a  $\beta$ -2,1-linked D-Frc polymer attached to a terminal D-Glc residue by  $\alpha$ -2,1-glycosidic bonds (Kelly, 2008) (Fig. 6). Based on its DP (2-60), it is classified as an oligo- or polyfructan (Mensink et al., 2015). Inulin occurs as a reserve carbohydrate in the tubers of flowering plants such as Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Cichorium intybus*), and dahlia

(*Dahlia pinnata*) and in the roots of dandelion (*Taraxacum officinale*) (Mohammed et al., 2011).

Chemical and biological decomposition of inulin leads to formation of Frc and oligofructans. The conversion to Frc is a single-step enzymatic reaction with 95% turnover (Vandamme & Derycke, 1983). As prebiotics, oligofructans positively affect on the growth of bifidobacteria and other beneficial microbes in the normal gastrointestinal flora, and they prevent the growth of pathogenic bacteria (Böhm et al., 2006).



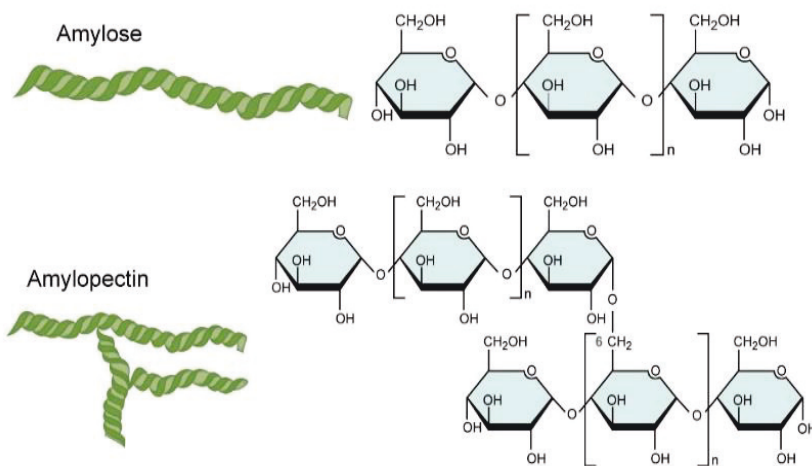
**Figure 6.** Inulin is composed of fructosyl subgroups linked together by  $\beta$ -2,1-glycosidic linkage attached to terminal D-glucosyl group by  $\alpha$ -2,1-glycosidic bond. Reprinted from Florian (2006) under public domain license. At: [https://commons.wikimedia.org/wiki/File:Inulin\\_struktur\\_formel.png](https://commons.wikimedia.org/wiki/File:Inulin_struktur_formel.png).

### 1.1.2.2. Starch

Starch is the major reserve carbohydrate, which is synthesized in green plant leaves and mobilized to the tubers, seeds, rhizomes and fruits where granular starch is accumulated and further stored as an energy source (Buleon et al., 1988; Martin & Smith, 1995; Blazek & Gilbert, 2011). It is considered as the

most common carbohydrate in human diets and can be generally found in foods such as potatoes, wheat, maize and rice. Native starches consist of two molecular components, namely amylose and amylopectin, which account for 20-30% and 70-80% of starch structure, respectively. Both consist of polymers of  $\alpha$ -D-Glc repeating units. In amylose these are linked by  $\alpha$ -(1 $\rightarrow$ 4)-, whereas in amylopectin about one residue in every twenty is also linked  $\alpha$ -(1 $\rightarrow$ 6)-, forming branching points (Li & Yeh, 2001) (Fig. 7).

Amylose is a linear molecule with few branching points, while amylopectin has a branched and compact structure (Gidley et al., 2010). In contrast to many structural polysaccharides, presence of  $\alpha$ -linked D-Glc repeating units, make amylose and amylopectin susceptible to chemical and enzymatic hydrolysis (Zobel, 1988; Hoover, 2001).



**Figure 7.** The molecular structure of amylose and amylopectin. Askiitians content team (2014). Reprinted from <http://www.askiitians.com> under askiitians foundation license.



## **1.2. Plant biomass-degrading enzymes**

Biodegradation of plant biomass polysaccharides to platform sugars is feasible by extracellular enzymes produced by filamentous fungi. According to the CAZy database, plant biomass polysaccharide-degrading enzymes and their subunits can be divided into the families of glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), carbohydrate binding modules (CBMs) and auxiliary activities (AAs) (Table 2) based on structural or sequence similarities (Lombard et al., 2014).

### **1.2.1. Enzymes involved in deconstruction of plant cell wall and storage polymers**

#### **1.2.1.1. Cellulolytic enzymes**

Cellulose degradation into platform sugars occurs via the cooperation of hydrolytic and oxidative enzymes. The hydrolytic enzymes can be divided into three main groups:

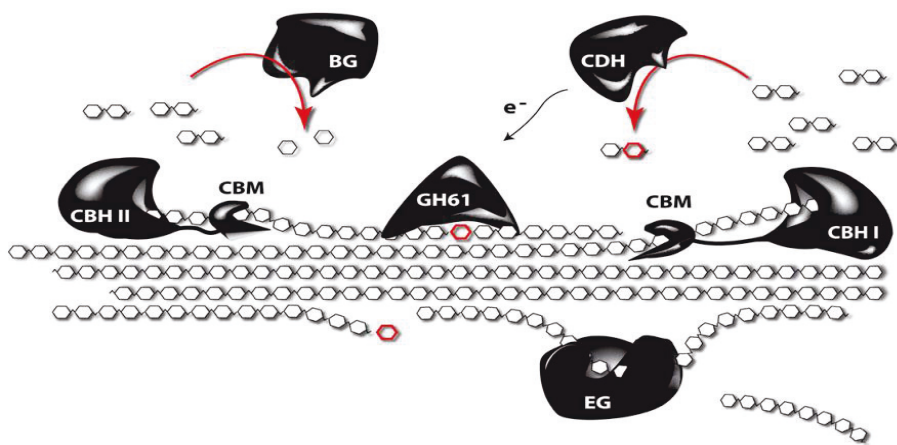
(i) endo- $\beta$ -1,4-glucanases (EGL) (EC 3.2.1.4), which randomly hydrolyse the glycosidic bonds within cellulose fibres

(ii) exo-glucanases or cellobiohydrolases (CBH I and CBH II; EC 3.2.1.176 and EC 3.2.1.91), which act on the reducing and non-reducing ends of cellulose

(iii)  $\beta$ -1,4-glucosidases (BGL) (EC 3.2.1.21) releasing monomeric glucose units from the resulting cellobiose (Bisaria & Ghose, 1981; Zeilinger et al., 2000) (Table 2).

The oxidative system involved in cellulose depolymerization is comprised of cellobiose dehydrogenases (CDHs; EC 1.1.99.18), in combination with lytic polysaccharide monooxygenases (LPMOs) (Philips et al., 2011). CDH plays

two proposed roles in enhancing cellulose depolymerization: (i) oxidative conversion of cellobiose and other short cello-oligosaccharides to related lactones (Ludwig et al., 2010), with concomitant electron production for LPMO catalytic activity (Sygmond et al., 2012); and (ii) the production of hydroxyl radicals through Fenton-type reactions, which participate directly in lignocellulose depolymerization (Mansfield et al., 1997) (Fig. 8).

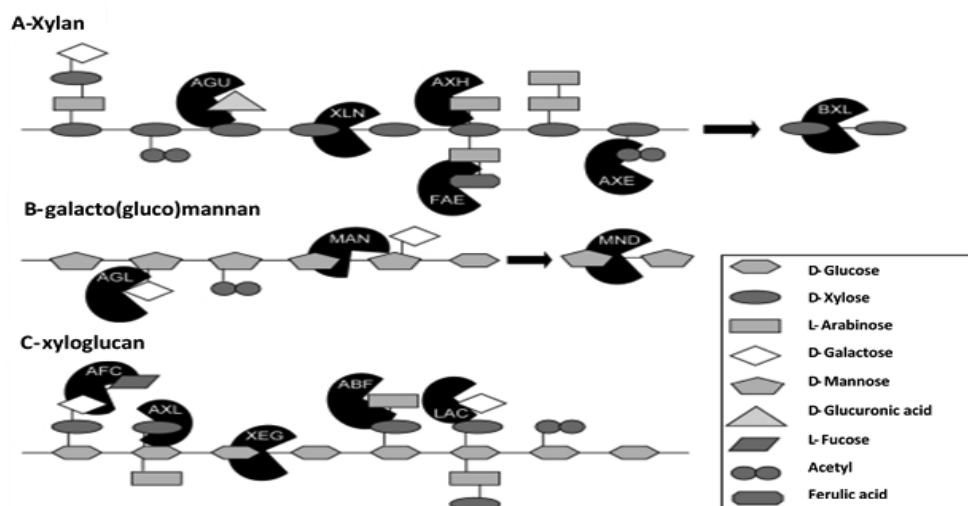


**Figure 8.** Schematic representation of cellulose decomposition by synergistic activity of oxidative and hydrolytic enzymes. BG:  $\beta$ -glucosidase, EG: endo-glucanase, CBH: cellobiohydrolase, CBM: cellulose binding domain, CDH: cellobiose dehydrogenase, GH61: glycoside hydrolase family 61, now LPMO. Reprinted from Vanholme et al. (2013) under the Creative Commons Attribution License (CC-BY 3,0).

LPMOs are copper-dependent enzymes (Johansen, 2016). They catalyse the oxidative cleavage of cellulose and  $\beta$ -(1  $\rightarrow$  4)-linked substituted and non-substituted glucosyl units of hemicellulose to form oxidized gluco-oligosaccharides (Frommhagen et al, 2015), which remarkably enhance the total hydrolysis.

### 1.2.1.2. Hemicellulolytic enzymes

Due to the complex structures of the various hemicelluloses, their degradation needs the synergistic activity of a set of enzymes to cleave diverse linkages within the hemicellulose backbone and side branch constituents (Fig. 9). The hemicellulose backbones are cleaved by endo- and exo-acting enzymes. The side branches can be hydrolysed either before or after the backbone cleavage (Tenkanen et al., 1996).



**Figure 9.** Schematic structure of three hemicelluloses A) xylan, B) galacto(gluco)mannan and C) xyloglucan, with hemicellulolytic enzymes acting on them. ABF, α-arabinofuranosidase; AFC, α-fucosidase; AGL, α-1,4-galactosidase; AGU, α-glucuronidase; AXE, acetyl (xylan) esterase; AXH, arabinoxylan α-arabinofuranohydrolase; AXL, α-xylosidase; BXL, β-1,4-xylosidase; FAE, feruloyl esterase; LAC, β-1,4-galactosidase; MAN, β-1,4-endomannanase; MND, β-1,4-mannosidase; XEG, xyloglucan-active β-1,4-endoglucanase; XLN, β-1,4-endoxylanase. Reprinted from van den Brink & de Vries (2011) with permission from Springer.

The xylan backbone is cleaved by  $\beta$ -1,4-D-endoxylanases ( $\beta$ -1,4-XLNs). The resulting xylo-oligomer chains are converted to D-Xyl monomers by  $\beta$ -1,4-D-xylosidase (BXL) activity (van Peij et al., 1997; Zhou et al., 2011). Feruloyl esterases (FAEs),  $\alpha$ -L-arabinofuranosidase (ABF), acetylxyylan esterases (AXE), and  $\alpha$ -1,4-D-galactosidase (AGL) cleave substituted FA, Ara, acetyl groups, and D-Gal, respectively, in the side branches of the xylan backbone (van den Brink & de Vries, 2011; Glass et al., 2013). The D-Glc repeating units of the xyloglucan backbone are cleaved by  $\beta$ -1,4-endoglucanase ( $\beta$ -1,4-EGL), and the side chains substituted with D-Gal, L-Ara, D-Xyl, and L-fucose are split by  $\beta$ -1,4-D-galactosidase ( $\beta$ -1,4-LAC), ABF,  $\alpha$ -D-xylosidase (AXL), and  $\alpha$ -fucosidase (AFC), respectively (Coutinho et al., 2009; van den Brink & de Vries, 2011; Glass et al., 2013) (Table 2).

Galactoglucomannan is decomposed by the joint action of  $\beta$ -1,4-D-endomannase ( $\beta$ -1,4-MAN),  $\beta$ -1,4-D-mannosidases ( $\beta$ -1,4-MND), and AGL (Pham et al., 2010). They catalyse the glycosidic bond hydrolysis within the mannan backbone releasing oligo- and di-Man and D-Gal substitutes (Coutinho et al., 2009; van den Brink & de Vries, 2011; Glass et al., 2013) (Table 2).

### **1.2.1.3. Lignin modifying enzymes**

Oxidative and nonspecific enzymes are involved in the decomposition of the complex, compact, and rigid structure of lignin (Kirk & Cullen, 1998; Hatakka & Hammel, 2010; Mäkelä et al., 2014). White-rot-causing basidiomycete fungi are known as the most efficient lignin modifiers. Aromatic lignin is degraded mainly by heme-peroxidases and laccases (Hatakka, 1994; Hammel & Cullen, 2008). Fungal heme-peroxidases such as lignin peroxidases (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs) belong to the plant peroxidase superfamily (Thurston, 1994; Hatakka, 1994; Martinez,

2002; Lundell et al., 2010). Laccases are extracellular multicopper enzymes that are able to oxidize polyphenols (Baldrian, 2006).

MnPs (EC 1.11.1.13) oxidize  $Mn^{2+}$  to  $Mn^{3+}$ .  $Mn^{3+}$  oxidizes the phenolic rings in lignin to phenoxy radicals, with subsequent ring opening. This leads to lignin solubilisation and mineralization (Wariishi et al., 1992; Gold et al., 2000). LiP (EC 1.11.1.14) catalyses the one-electron oxidation of both phenolic and non-phenolic aromatic compounds, resulting in the oxidative cleavage of  $C_{\alpha}$ - $C_{\beta}$ , leading to ring opening and decomposition of lignin (Lundell et al., 1993; Hatakka, 1994). VPs (EC 1.11.1.16) are hybrid peroxidases that structurally resemble LiPs, and their catalytic specificities are similar to those of MnPs (Martinez, 2002).

Laccases (EC 1.10.3.2) are involved in the biodegradation of wood and lignocellulosic materials, as well as in pigmentation, fruiting body formation and sporulation (Langfelder et al., 2003; Baldrian, 2006). *Sensu stricto* laccases are mainly produced by white-rot basidiomycetes, but laccase-like multicopper oxidases, which are produced by plants, bacteria, archaea and insects have also been reported (Giardina et al., 2010). Laccases catalyse the reduction of oxygen to water, with concomitant oxidation of phenolic rings that leads to phenoxy radical generation and ring opening (Baldrian, 2006). Non-phenolic compounds can also be oxidized by laccases if mediators, such as vanillin are available (Pardo & Camarero, 2015).

#### **1.2.1.4. Feruloyl esterases (FAEs)**

FAEs (EC 3.1.1.73) are also known as ferulic, cinnamic or cinnamoyl acid esterases, which belong to a sub-class of carboxylesterases (Bonnina et al., 2001; Shin & Chen 2006; Benoit et al., 2008). Together with acetylxytan esterases, FAEs are accessory biocatalysts involved in releasing cinnamic acid

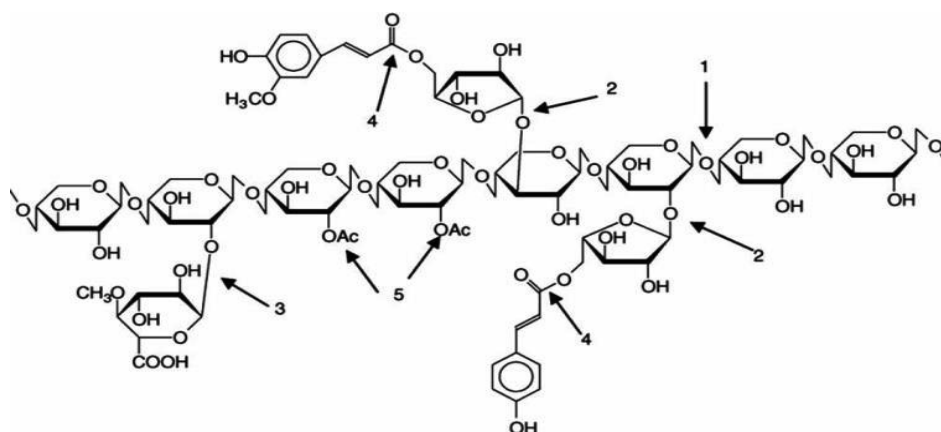
derivatives from the plant cell wall, which assists the hemicellulolytic and pectinolytic systems (Williamson et al., 1998; de Vries & Visser, 2001; Panagiotou et al., 2007; Zhang et al., 2015) (Fig. 10). FAEs cleave the diferulate cross-linkage between adjacent xylan chains as well as the xylan chains and lignin, enhancing the digestibility of the lignocellulosic materials (Sun et al., 2002; de O. Buanafina, 2009; Scheller & Ulvskov, 2010). Furthermore, they can release small hydroxycinnamates, such as the chlorogenic acid (caffeic acid-quinic acid) found in coffee beans (Juge et al., 2001). Consequently, they facilitate efficient depolymerization of plant cell wall polysaccharides (Yu et al., 2002; Tabka et al., 2006). In addition to their hydrolytic activities, FAEs contribute to the formation of bioactive components, such as vanillin, which is one of the most commonly used aromatic molecules in the food industry (Topakas et al., 2007).

FAEs were first classified, based on their substrate profiles (Kroon et al., 1999). The classification has since been extended into four subclasses (A, B, C, and D), based on their amino-acid sequence identity and substrate specificities (Crepin et al., 2004). Recently, phylogeny-based analysis has broadened FAE classification (Dilokpimol et al., 2016).

FAEs have a conserved active-site catalytic triad (serine-histidine-aspartic acid) and show a broad range of substrate specificities (Kroon et al., 1997; Topakas et al., 2005; Udatha et al., 2012). FAEs are active towards synthetic hydroxycinnamate methyl esters such as methyl caffeate (MCA), methyl *p*-coumarate (MpCA), methyl ferulate (MFA), and methyl sinapinate (MSA) (Crepin et al., 2004). Type-A FAEs favour ferulic and sinapinic acids, where they act on an aromatic moiety that is substituted by methoxy groups at the *meta* position (Topakas et al., 2007). Type-B FAEs favour *p*-coumaric or caffeic acids, which contain one or two methoxy substitutions. Furthermore,

the low amounts of diferulates are released by type-A and D FAEs. Type-C and D show broad substrate specificity towards ferulic, *p*-coumaric as well as sinapinic acids and are able to release diferulates (Topakas et al., 2005, 2007; Gopalan et al., 2015). FaeA and FaeB of *A. niger* are the best-characterized FAEs (de Vries et al., 1997, 2002).

Ascomycetous fungi such as *Aspergillus* species (de Vries et al., 1997; Asther et al., 2002; Mathew & Abraham, 2004; Koseki et al., 2005), *Penicillium* species (Topakas et al., 2007) and the thermophilic fungus *Sporotrichum thermophile* (Topakas et al., 2003) are well-known FAE producers. They produce FAEs in the presence of ferulic acid esters, mainly methyl and ethyl or hydroxycinnamic acid-rich plant cell wall substrates such as wheat bran (WB), oat spelt xylan, sugar beet pulp (SBP) and maize bran (de Vries et al., 1997; Faulds et al., 1997; de Vries & Visser, 1999; Topakas et al., 2003).



**Figure 10.** Schematic representation of xylan structure and xylanolytic enzyme system. 1) Endo-xylanases; 2)  $\alpha$ -L-arabinofuranosidases; 3)  $\alpha$ -glucuronidases; 4) feruloyl and coumaroyl esterases; 5) acetyl xylan esterases. Reprinted from Chavez et al. (2006) with permission from Elsevier.

While plant polysaccharide-hydrolysing enzymes mostly belong to the GHs in the CAZy database (Lombard et al., 2014), FAEs are classified in the CE1 family, which is related to the acetyl xylan esterases (AXEs), with few exceptions. However, not all FAEs are classified in the CAZy database. These include *A. niger* FaeA and FaeB, which have sequence similarity to lipases and tannases, but without detectable lipase- and tannase-related activities (de Vries et al., 2000, 2002; Benoit et al., 2008).

### 1.2.1.5. Pectinolytic enzymes

Pectinases are a heterogeneous group of enzymes that depolymerize (hydrolases and lyases) and de-esterify (esterases) pectic substances present in the plant cell wall (Blanco et al., 1999; Pedrollo et al., 2009; Yadav et al., 2009; Saranraj & Naidu, 2014). Pectinases are among the most widely distributed enzymes in fungi, bacteria and plants (Tapre & Jain, 2014). They are classified in three main classes: (i) protopectinases, (ii) depolymerizing and cleaving enzymes and (iii) pectin esterases, based on substrate specificity and mode of activity (Pandey, 1992; Sakai et al., 1993). Their classification and activities are presented in Table 1.

**Table 1.** Classification of pectinolytic enzymes based on their modes of activity and preferred substrate.

Type of pectinases	EC number	CAZy family	Substrate	Mode of action	Product
1. Esterases					
PME	3.1.1.11	CE8	Pectin	Hydrolysis	Pectic acid + methanol
PAE	3.1.1.6	CE12, CE13	Pectin	Hydrolysis	Pectic acid + acetate
2. Depolymerases					
a) Hydrolases					
PGA	3.2.1.15	GH28	Pectic acid	Hydrolysis	Oligogalacturonates
PGX	3.2.1.67	GH28	Pectic acid	Hydrolysis	Monogalacturonates
PMG	3.2.1.-		Pectin (SR)	Hydrolysis	Oligomethylgalacturonate, methylgalacturonate
RG-Rhamnohydrolase	3.2.1.40	GH28, GH78, GH106	Pectin (HR)	Hydrolysis	L-Rhamnose
RG-Galacturonohydrolase	3.2.1.-	GH28	Pectin (HR)	Hydrolysis	Monogalacturonate



RG-Hydrolase			Pectin (HR)	Hydrolysis	Oligogalacturonate
RGL	4.2.2.-		Pectin (HR)	Transelimination	
RGAE	3.1.1.-	GH12	Pectin (HR)	Hydrolysis	Rhamnogalacturonan chain
XGA-Hydrolase	3.2.1.-	GH28	Pectin (HR)	Hydrolysis	Xylose-galacturonate dimer
RHG					
RGX					
b) Lyases					
Endo-PLY	4.2.2.2	PL1, PL3, PL9	Pectic acid	Transelimination	Unsaturated oligogalacturonates
Exo-PYL	4.2.2.9	PL1, PL3, PL9	Pectic acid	Transelimination	Unsaturated oligogalacturonates
Endo-PEL	4.2.2.10	PL1	Pectin (SR)	Transelimination	Unsaturated methyloligo-galacturonate
PGL	4.2.2.2	PL1, PL2, PL3	Pectic acid	Transelimination	Unsaturated oligogalacturonates
c) Accessory enzymes					
					-
ABF	3.2.1.55	GH54, GH51, GH54	Pectin (HR)	Hydrolysis	-
LAC	3.2.1.23	GH2,	Pectin (HR)	Hydrolysis	-
FAE	3.1.1.73	CE1	Pectin (HR)	Hydrolysis	-
<i>p</i> -coumaroyl esterase		CE1	Pectin (HR)	Hydrolysis	-
BXL	3.2.1.37	GH3, GH39, GH43	-	-	-
GAL	-	-	-	-	-
ABN	3.2.1.99	GH43	-	-	-
ABX	3.2.1.-	GH93	-	-	-
XGH	3.2.1.-	GH28	-	-	-
XGX	-	-	-	-	-

ABF:  $\alpha$ -arabinofuranosidase, ABN: endo-arabinanase, ABX: exo-arabinanase, BXL:  $\beta$ -1,4-xylosidase, CE: carbohydrate esterase, FAE: feruloyl esterase, GAL:  $\beta$ -1,4-galactanase, GH: glycoside hydrolase, HR: hairy region, LAC:  $\beta$ -galactosidase, PAE: pectin acetyl esterase, PEL: pectin lyase, PG: polygalacturonase, PGA: endo-polygalacturonase, PGL: polygalacturonate lyase, PGX: exo-polygalacturonase, PL: polysaccharide lyase, PLY: pectate lyase, PME: pectin methyl esterase, PMG: polymethyl-galacturonase, RG: rhamnogalacturonan, RGAE: rhamnogalacturonan acetyl esterase, RGL: rhamnogalacturonan lyase, RGX: exo-rhamnogalacturonase, RHG: endo-rhamnogalacturonase, SR: smooth region, XGA: xylogalacturonan, XGH: xylogalacturonan hydrolase, XGX: exo-xylogalacturonase (based on de Vries & Visser, 1999; de Vries & Visser, 2001; Yadav et al., 2009; van den Brink & de Vries, 2011).

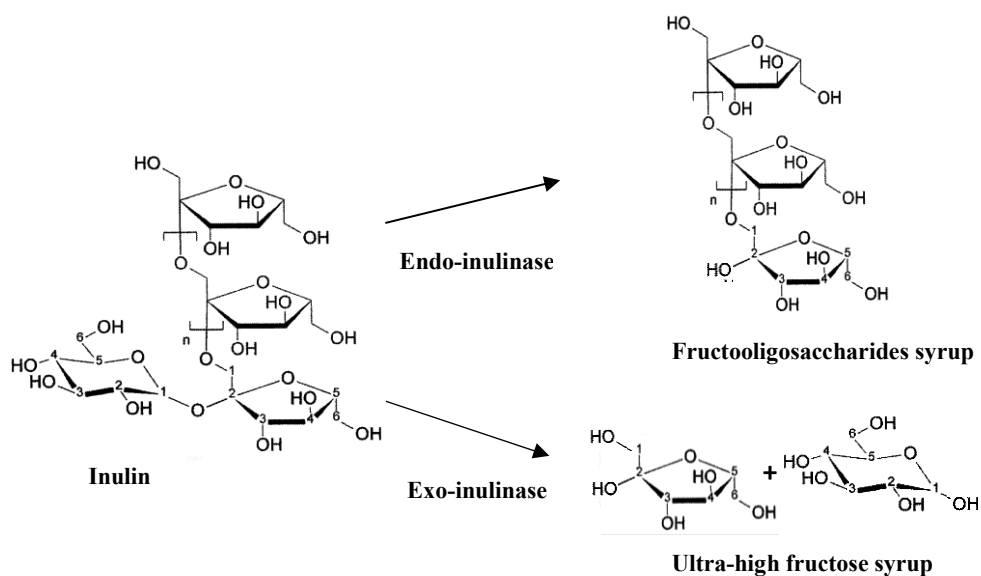
## 1.2.2. Enzymes involved in depolymerization of plant storage polymers

### 1.2.2.1. Inulinases

Inulinases ( $\beta$ -2,1-D-fructan fructanohydrolases) are extracellular enzymes that break down  $\beta$ -2,1-glycosidic bonds within inulin chains (Ettalibi & Baratti,

2001; Gong et al., 2008; Abu El-Souod et al., 2014). Endo-inulinases (INUs) ( $\beta$ -2,1-D-fructan fructanohydrolase; EC 3.2.1.7), which randomly cleave the glycosidic bonds within the inulin chain (Fig. 11), release tri-, tetra- and penta-inulo-oligosaccharides (Ettalibi & Baratti, 2001; Ohta et al., 2002). Exo-acting inulinases ( $\beta$ -D-fructohydrolase; EC 3.2.1.80) cut sucrose-like glycosidic bonds from the non-reducing end of the inulin chain (Fig. 11), leading to the release of a Frc unit (Vandamme & Derycke, 1983; Chi et al., 2009). Furthermore, levan- (polyfructan) and sucrose-acting enzymes can be categorized as levanases and sucrases/invertases (SUCs) based on the hydrolyzation of the corresponding substrate (Vijayaraghavan et al., 2009).

Numerous microorganisms (Ricca et al. 2007; Nascimento et al., 2012) are able to depolymerize inulin. Filamentous fungi, mainly *Aspergillus* and *Penicillium* species, are well-known inulinase producers (Liu et al., 2013; Rawat et al., 2015b). Many endo- and exo-acting inulinases from *Penicillia* have been isolated, identified and characterized including those from *Penicillium trzebinskii* (Onodera & Shiomi, 1992), *Penicillium* sp. TN-88 (Nakamura et al., 1997), *Penicillium aculeatum*, *Penicillium digitatum*, *Penicillium cyclopium* (Singh & Gill, 2006), *Penicillium citrinum* (Adriana et al., 2012) and *Penicillium rugulosum* (Rawat et al., 2015a). Moreover, *P. rugulosum* and *P. purpurogenum* which were isolated from dahlia tubers (Barthomeuf et al., 1991) and the chicory rhizosphere, showed high inulinase production capacity, and the latter illustrated an increase in enzyme activity after chemical mutagenesis (Sharma & Gill, 2007).



**Figure 11.** Schematics of the action of endo-inulinase (INU) or exo-inulinase (INX) on inulin, to produce fructo-oligosaccharide syrup or ultra-high fructose syrup respectively. Modified from Fernandes (2014).

Frc and fructooligosaccharides are the main industrial products of inulinases (Gill et al., 2006; Chi et al., 2011). Frc which is industrially used for production of HFS is produced by a single-step inulolytic process (95% yield) that is more efficient than starch hydrolysis using enzyme mixtures (45% yield) (Vandamme & Derycke, 1983; Chi et al., 2011; Kango & Jain, 2011; Liu et al., 2013). *Penicillium* species are promising industrial producers of both endo- and exo-acting inulinases. The high temperature optima and thermostability of some of these enzymes may make them suitable for industrial purposes (Singh & Gill, 2006).

#### 1.2.2.2. Amylases

Enzymes involved in starch depolymerization have a wide range of applications in the food, textile, and paper industries (Pandey et al., 2000a).

They are a group of hydrolytic enzymes that act synergistically converting starch to Glc units (Gupta et al., 2003). Amylases are plant-, animal- and microbe-originated enzymes (Pandey et al., 2000b). Microbial amylases, particularly fungal enzymes, are important for industrial purposes, because they have GRAS status (Hernández et al., 2006; Kathiresan & Manivannan, 2006).

Starch-degrading enzymes are classified as: (i) endo-amylases, (ii) exo-amylases and (iii) debranching enzymes (van der Maarel et al., 2002). Endo-amylases (EC 3.2.1.1), e.g.  $\alpha$ -amylases (AMYs) randomly split  $\alpha$ -1,4-glycosidic bonds within amylose and amylopectin chains, resulting in the liberation of linear and branched oligo- and monosaccharides (Gupta et al., 2003). Exo-acting amylases catalyse the hydrolysis of  $\alpha$ -1,4-glycosidic bonds ( $\beta$ -amylase, EC 3.2.1.2), or  $\alpha$ -1,6-glycosidic (amyloglycosidase, EC 3.2.1.3, and  $\alpha$ -1,6-glucosidase, EC 3.2.1.20) at the nonreducing end of the amylose and amylopectin chain to release maltose,  $\beta$ -limit dextrin and Glc.

**Table 2.** Fungal enzymes involved in degradation of lignin, and plant structural and storage polysaccharides.

Substrate	Enzyme abbreviation	Enzyme class	EC number	CAZy families
Cellulose	EGL	$\beta$ -1,4-D-Endoglucanase	3.2.1.4	GH5, 7, 9,12, 44, 45
	CBH	Cellobiohydrolase	3.2.1.91	GH6, 7
	BGL	$\beta$ -1,4-D-Glucosidase	3.2.1.21	GH1, 3
Xylan	AGU	$\alpha$ -Glucuronidase	3.2.1.51, 3.2.1.139	GH67, 115
	AXE	Acetyl xylan esterase	3.2.1.72	CE1
	AXH	Arabinoxylan arabinofuranohydrolase	3.2.1.55	GH62
	GE	Glucuronoyl esterase	3.1.1.-	CE15
	ABF	$\alpha$ -L-Arabinofuranosidase	3.2.1.55	GH51, 54
	AGL	$\alpha$ -1,4-D-Galactosidase	3.2.1.22	GH27 36
	FAE	Feruloyl esterase	3.1.1.73	CE1
	BXL	$\beta$ -1,4-D-Xylosidase	3.2.1.37	GH3 39 43
	LAC	$\beta$ -1,4-D-Galactosidase	3.2.1.23	GH2, 35, 42
	XLN	$\beta$ -1,4-D-Endoxylanase	3.2.1.8	GH10, 11
Xyloglucan	ABF	$\alpha$ -L-Arabinofuranosidase	3.2.1.55	GH51, 54
	AFC	$\alpha$ -L-Fucosidase	3.2.1.51, 3.2.1.63	GH29, 95
	AGL	$\alpha$ -1,4-D-Galactosidase	3.2.1.22	GH27, 36
	AXL	$\alpha$ -D-Xylosidase	3.2.1.77	GH31

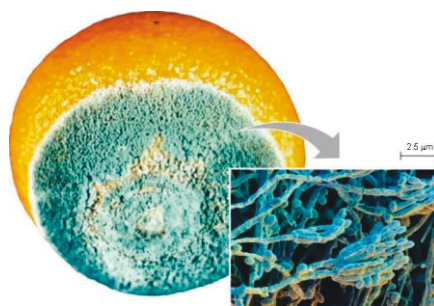
	CBH	Cellobiohydrolase	3.2.1.91	GH6, 7, 9
	EGL	$\beta$ -1,4-D-Endoglucanase	3.2.1.4	GH5, 7, 9, 12, 44, 45
	LAC	$\beta$ -1,4-D-Galactosidase	3.2.1.23	GH2, 35, 42
	XGAE	Xyloglucan acetyl esterase	3.1.1.-	-
	XEG	Xyloglucan-active $\beta$ -1,4-D-endoglucanase	3.2.1.151	GH12, 74
Galact(gluco)mannan	AGL	$\alpha$ -1,4-D-Galactosidase	3.2.1.22	GH27 36
	GMAE	Galactomannan acetyl esterase	3.1.1.-	-
	LAC	$\beta$ -1,4-D-Galactosidase	3.2.1.23	GH2, 35, 42
	MAN	$\beta$ -1,4-D-Endomannase	3.2.1.78	GH5, 26
	MND	$\beta$ -1,4-D-Mannosidase	3.2.1.25	GH2
	BGL	$\beta$ -1,4-D-Glucosidase	3.2.1.21	GH1, 3
Lignin	LiP	Lignin peroxidase	1.11.1.14	AA2
	MnP	Manganese peroxidase	1.11.1.13	AA2
	VP	Versatile peroxidase	1.11.1.16	AA2
		Laccase	1.10.3.2	AA1
Pectin	ABN	Endo-arabinanase	3.2.1.99	GH43
	ABX	Exo-arabinanase	3.2.1.-	GH93
	GAL	$\beta$ -1,4-Endogalactanase	3.2.1.89	GH53
	GLN	$\beta$ -1,6-Endogalactanase	3.2.1.164	GH5, 30
	LAC	$\beta$ -1,4-D-Galactosidase	3.2.1.23	GH2, 35, 42
	PAE	Pectin acetyl esterase	3.2.1.-	-
	PEL	Pectin lyase	4.2.2.10	PL1
	RGL	Rhamnogalacturonan lyase	4.2.2.-	PL4, 11
	PGA	Endo-polygalacturonase	3.2.1.15	GH28
	PGX	Exo-polygalacturonase	3.2.1.82	GH28
	PLY	Pectate lyase	4.2.2.2	PL1, 3, 9
	PME	Pectin methyl esterase	3.1.1.11	CE8
	RGAE	Rhamnogalacturonan acetyl esterase	3.1.1.-	CE12
	RGX	Exo-rhamnogalacturonase	3.2.1.-	GH28
	RHA	$\alpha$ -Rhamnosidase	3.2.1.40	GH78
	RHG	Endo-rhamnogalacturonase	3.2.1.171	GH28
	UGH	d-4,5-Unsaturated-glucuronyl hydrolase	3.2.1.-	GH88
	URH	Unsaturated rhamnogalacturonan hydrolase	3.2.1.172	GH105
	XGH	Xylogalacturonan hydrolase	3.2.1.-	GH28
	XTG	$\beta$ -1,3-Exogalactanase	3.2.1.145	GH43
Inulin	INU	Endo-inulinase	3.2.1.7	GH32
	INX	Exo-inulinase	3.2.1.80	GH32
	SUC	Invertases	3.2.1.26	GH32
Starch	AGD	$\alpha$ -1,4-D-Glucosidase	3.2.1.20	GH31
	AMY	$\alpha$ -Amylase	3.2.1.1	GH13
	GLA	Glucoamylase	3.2.1.3	GH15

Adapted and modified according to de Vries & Visser 2001, van der Maarel et al., 2002; Gong et al., 2008; Coutinho et al., 2009, van den Brink & de Vries 2011, Mäkelä et al., 2014, Rytioja et al., 2014.

### 1.3. Industrially important ascomycetes: prevalence, characteristics and biotechnological applications of *Penicillium* and *Aspergillus*

#### 1.3.1. Genus *Penicillium*

The genus *Penicillium* includes many significant ascomycetous fungi from the environmental, industrial, and medical perspectives. It was initially described by Johann Heinrich Friedrich Link in 1809 as a fungal family that produces brush like asexual proliferative structures called ‘penicilli’ and introduced *Penicillium expansum* Link as the type species reviewed by Samson and Pitt (1985) and Haubrich (2003). Penicillia are considered as the most prevalent filamentous fungi among the eukaryotic microorganisms, with over 350 described species (Pitt, 1980; Kirk et al., 2008; Visagie et al., 2014; Frisvad, 2015). Ubiquitous *Penicillium* species are well-known as blue and green moulds that commonly invade citrus fruits, of which some examples are *Penicillium digitatum* and *Penicillium italicum*. They apply a powerful enzyme cocktail to degrade and convert the plant cell wall components. A brush-like asexual spore-bearing structure is the unique feature of the genus and is used as a tool to allocate Penicillia to the correct species.



**Figure 12.** *Penicillium* sp. colonizes citrus fruit with brush like conidiophores-bearing conidia (Rose Yao, [www.pinterest.com](http://www.pinterest.com)).

### 1.3.1.1. Ecology of *Penicillia*

*Penicillium* species are opportunistic saprobes and are common contaminants in foods, feeds, drinks and indoor surroundings and widely present in soil ecosystems (Banke et al., 1997; Samson et al., 2004). They successfully colonize soil environments through their simple nutritional needs and growth competence. They produce a wide range of active biomolecules that influence the life cycles of soil inhabitants (Khalid et al., 2006). *Penicillia* survive by absorbing nutritional elements from plant biomass material and biodegradable organic substances using various extracellular enzymes (Panda, 2011). These enzymes are also able to degrade xenobiotic compounds leading to soil-pollutant transformation and elimination (Leita, 2009).

### 1.3.1.2. Production of macromolecules and secondary metabolites

The genus *Penicillium* is prominent for the production of medically important extrolites (secondary metabolites) of bactericidal, antiviral, antifungal and immunosuppressant activities (Table 3). This is seemingly associated with their ability to compete for nutritional elements in their natural habitats.

**Table 3.** The most common medically important secondary metabolites produced by *Penicillia*.

Secondary metabolites	Producer(s)	Activity	Reference
Fulvic acid	<i>P. brefeldianum</i>	Antifungal, antiviral	Kurobane, 1981
Patulin	<i>P. griseofulvum</i>	Antibacterial	Jimenez, 1988
Griseofulvin	<i>P. griseofulvum</i> , <i>P. janczewskii</i>	Antifungal	De Carli, 1988
Penicillin	<i>P. nalgiovense</i> , <i>P. chrysogenum</i> <i>P. rubens</i>	Antibacterial	Andersen & Frisvad, 1994 Houbraken et al., 2011a
Mycophenolic acid	<i>P. brevicompactum</i>	Immunosuppressant	Bentley, 2000
1,8-O-Dimethylaverantin	<i>P. chrysogenum</i>	Antifungal	Maskey et al., 2003
8-O-Methylaverufin	<i>P. chrysogenum</i>	Antifungal	Maskey et al., 2003
Mevastatin	<i>P. citrinum</i>	Cholesterol lowering agent	Ahmad et al., 2010

Penicillin was first detected by Alexander Fleming in 1929 in a culture of *Staphylococcus aureus* contaminated by *Penicillium rubrum*, which was subsequently re-identified and described as *Penicillium notatum*, synonym of *Penicillium chrysogenum*/*Penicillium rubens* (Houbraken et al., 2011a). The discovery of penicillin was a revolutionary milestone in the production of antibiotics and other therapeutics (Davies & Davies, 2010). Penicillia are also producers of numerous toxic products termed mycotoxins that are harmful to plants, animals and humans (Frisvad & Filtenborg, 1983; Nielsen, 2003). Important mycotoxins include penitrem A produced by *Penicillium crustosum*, cyclopiazonic acid, cyclopiamine and cyclopimide produced by *Penicillium griseofulvum*, in addition to ochratoxin and citrinin which are produced by *Penicillium verrucosum* (Frisvad, 1989; Houbraken et al., 2014).

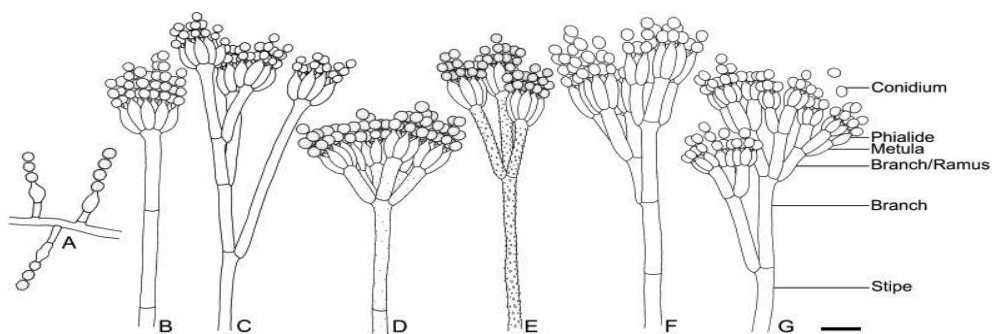
### **1.3.1.3. Identification**

Penicillia are recognizable by brush-like conidium-bearing structures called penicilli (sing.: penicillus), which are significant tools for identification and classification of the genus (Pitt, 1979; Haubrich, 2003). The stalks are called conidiophores, which are either hyaline, smooth or rough-walled. They may also be simple or branched, decorated by clusters of flask-shaped structures called phialides. The typically green-coloured and dry chains of conidia are germinated from the head of the phialides, where the youngest conidium is situated at the bottom of the conidial chain (Fig. 13).

There are several micro-morphological specificities used in the identification of the genus *Penicillium*. In addition to the characteristics such as cell-wall texture, ornamentation of stalks and conidia, dimensions, arrangements and colours of the conidiophore segments, conidiophore branching patterns are important in identification (Pitt, 1979). They show a



wide range of patterns varying from simple (singular) phialides to highly complex ones. The most recognized patterns are: (i) non-branched or monoverticillate, a group of phialides at the top of the stipe, (ii) branched (symmetrical and asymmetrical) comprised of (a) divaricate: simple to complex branching arrangements with several sub-terminal branches, (b) biverticillate: a cluster of branches, each bearing a cluster of phialides, (c) terverticillate: branches of conidiophores followed by a second order of branches carrying a cluster of phialides and (d) quaterverticillate conidiophores with one additional level of branching beyond the terverticillate pattern (Fig. 13) (Visagie et al., 2014).



**Figure 13.** Conidiophore branching patterns observed in *Penicillium*. A) Conidiophores with singular phialides, B) Monoverticillate, C) Divaricate, D) and E) Biverticillate, F) Terverticillate, G) Quaterverticillate. Reprinted from Visagie et al. (2014) with permission from Westerdijk Fungal Biodiversity Institute.

#### 1.3.1.4. Taxonomy

The first taxonomic study of the *Penicillia* was published in 1930. Raper and Tom (1949) classified the genus *Penicillium* into certain species, based on the combination of macroscopic characteristics, such as colony texture and colour, along with morphological features. Later, based on micro- and macro-

morphological characteristics, such as conidiophore morphology and branching patterns, the genus *Penicillium* was divided into four subgenera, which were *Aspergilloides*, *Biverticillium*, *Furcatum*, and *Penicillium* (Pitt, 1979). Due to strain variations that led to confusing taxonomic results, the proposed classification was accepted but debatable (Grimm et al., 2005).

To obtain a more accurate and acceptable classification, information on the production of different biologically active metabolites, such as alkaloids, antibiotics and mycotoxins was used to describe the *Penicillia* (Ismaiel & Papenbrock, 2014). In addition, a novel polyphasic taxonomy was adopted, in which the secondary metabolites profiles together with micro- and macro-morphological features were used for species typing (Samson et al., 2006).

Despite improved classification criteria there were still uncertainties in the taxonomic studies on the genus *Penicillium* since the strains from the same species did not show equal features or profiles (Frisvad & Samson, 2004; Redondo et al., 2009). Advanced and innovative molecular techniques of high precision and accuracy, such as sequencing of housekeeping genes, as well as ribosomal and mitochondrial DNA, have been developed and exploited. In particular, to assign *Penicillium* sp. to a certain *Penicillium* species,  $\beta$ -tubulin (BenA), calmodulin (CaM), RNA polymerase II (RPB2) as well as ITS (internal transcribed spacer) regions have been sequenced (Houbraken et al., 2014; Visagie et al., 2014; Yilmaz et al., 2014). Accordingly, the genus *Penicillium* is assigned to the family *Aspergillaceae*, order *Eurotiales*, class *Eurotiomycetes*, and the phylum *Ascomycota* (Houbraken & Samson, 2011).

### 1.3.1.5. Biotechnological potential

The genus *Penicillium*, which is comprised of over 300 species, plays important roles in biotechnological applications. Their potential utilizations range from bioremediation, low-cost production of antioxidant, vitamin, pigment, antibiotic and antifungal agents to the production of several classes of enzymes (Sharaf Ali et al., 2016). The enzymes are used in the food, beverages and feed, textile and detergent, pulp and paper, biorefinery and biofuels, wine and brewery industries and in the extraction of olive oil and pigments (Kuhad et al., 2011; Menendez et al., 2015).

In the food-processing industries, *Penicillium camemberti* and *Penicillium roqueforti* are used to improve the flavour of processed cheeses such as Camembert and Roquefort. Furthermore, *Penicillium nalgiovense* is used in surface-ripened sausages to enhance taste and prevent contamination by opportunistic microorganisms (Geisen et al., 2001; Leroy et al., 2006). In addition, cellulases, hemicellulases, pectinases, proteases, amylases, lipases, inulinases, FAEs and chitinases are among the most important biotechnological and industrial enzymes produced by *Penicillia* (Table 4) (Guimarães et al., 2006).

**Table 4.** Biotechnologically and industrially important enzymes produced by *Penicillia*.

Enzymes	Potential biotechnological and/or industrial applications	Producer(s)	References
Proteases	Food processing, beverage, textile, pulp and paper, detergent, feed, leather making and medicinal industries.	<i>P. citrinum</i> <i>P. camemberti</i> <i>P. roqueforti</i> <i>P. chrysogenum</i> <i>P. chrysogenum</i>	Yamamoto et al., 1993 Chrzanowska et al., 1995 Larsen et al., 1998 Afifi et al., 2014 Ikram-Ul-Hag & Mukhtar, 2006
Lipases	Detergent, dairy, food, bakery, beverage, chemical, cosmetic, pharmaceuticals, leather, and paper industries, meat and fish, and fat and oil processing.	<i>P. citrinum</i> <i>P. roqueforti</i> <i>P. restrictum</i> <i>P. camemberti</i> <i>P. cyclopium</i>	Sztajer & Maliszewska, 1989; D'Annibale et al., 2006 Petrovic et al., 1990 Gombert et al., 1999 Ghosh et al., 1996 Chahinian et al., 2000
Amylases	Production of fructose and glucose by enzymatic conversion of starch, textile desizing, baking, detergent, paper, and biofuel production industries.	<i>P. fellutanum</i> <i>P. purpurogenum</i> <i>P. chrysogenum</i> <i>P. janthinellum</i> <i>P. expansum</i>	Kathiresan & Manivannan, 2006 Silva et al., 2011 Balkan et al., 2005 Sindhu et al., 2009 Erdal et al., 2010
Pectinases	Fruits and vegetable, textile, wine, and animal feed processing, tea and coffee fermentations, extraction of vegetable oils, biobleaching of kraft pulp, poultry feed productions and recycling of waste papers.	<i>P. italicum</i> <i>P. oxalicum</i> <i>P. veridicatum</i> <i>P. chrysogenum</i> <i>P. citrinum</i>	Alaña et al., 1990 Yadav & Shastri et al., 2007 Silva et al., 2002 Banu et al., 2010 El-Batal et al., 2013
Xylanases	Pulp biobleaching, elimination of lignin residues from kraft pulp, increasing animal feed digestibility, beverage industries, production of biochemicals and biofuels from lignocellulose, food, and textile industries.	<i>P. brasilianum</i> <i>P. funiculosum</i> <i>P. janthinellum</i> <i>P. verruculosum</i> <i>P. simplicissimum</i> <i>P. janczewskii</i> <i>P. funiculosum</i>	Thygesen et al., 2003 Mishra et al., 1984 Curotto et al., 1994 Berlin et al., 2000 Schmidt et al., 1998 Terrasan et al., 2010 Alcocer et al., 2003
Chitinases	Antimicrobial and insecticide agent, bio-conversion of chitin to active pharmaceutical, and single cell protein production.	<i>P. aculeatum</i> <i>P. janthinellum</i> <i>P. ochrochloron</i>	Binod et al., 2005 Fenice et al., 1998a Patil & Jadhav, 2015
Cellulases	Pulp and paper, textile (bio-stoning and biopolishing), biorefinery and biofuel, wine and brewery, and agricultural industries food and feed processing, extraction of olive oil and pigments, medical applications and waste management.	<i>P. brasilianum</i> <i>P. echinulatum</i> <i>P. verruculosum</i> <i>P. decumbens</i> <i>P. oxalicum</i> <i>P. glabrum</i> <i>P. chrysogenum</i>	Jørgensen et al., 2003 Rubini et al., 2010 Morozova et al., 2010 Chen et al., 2010 Copa-Patiño et al., 1990 Knob et al., 2013 Hou et al., 2007

Feruloyl esterases	Extraction of hydroxycinnamic acids such as ferulic acids from natural sources, supplementation of digestive enzymes, food processing, biofuels, pharmaceutical and pulp and paper industries.	<i>P. funiculosum</i> <i>P. brasilianum</i> <i>P. expansum</i> <i>P. brevicompactum</i>	Kroon et al., 2000 Panagiotou et al., 2007 Donaghy & McKay, 1995 Donaghy & McKay, 1995
Inulinases	Ultra-high fructose syrup and inulo-oligosaccharides production with a wide variety applications in food and pharmaceutical industries; biorefinery and biofuel industries. Single-cell oil and single-cell protein production.	<i>P. rugulosum</i> <i>P. funiculosum</i> p.36 <i>P. citrinum</i> <i>P. janthinellum</i> <i>P. purpurogenum</i> <i>Penicillium</i> sp. TN-88	Barthomeuf et al., 1991 Danial et al., 2015 Lafuente-Castaneda, 2012 Wang et al., 2011 Muthuselvi et al., 2012 Nakamura et al., 1997

### 1.3.2. Genus *Aspergillus*

The genus *Aspergillus* is a group of filamentous fungi belonging to the family Trichocomaceae within the phylum Ascomycota. It was initially described in 1729 by Micheli, an Italian priest and biologist, in his work ‘*the Nova Geneva Plantarum*’ (de Vries & Visser, 2001). He named the genus according to the microscopic form of the spore-bearing structures that looked like an aspergillum, the holy water sprinkler (Bennett, 2010; Prakash & Jjha, 2014).

The major taxonomic feature of *Aspergillus* species is the asexual aspergillum-like conidium-forming structures (Machida & Gomi, 2010). The genus is classified into species, based on the combined data obtained from morphological and biochemical analysis and molecular techniques (de Vries & Visser, 2001).

The hyphae and colour of the conidium, size of the colony and colony reverse colour, exudate and pigment production as well as growth rate, are among the most important macro-morphological characteristics used in identification. The main micro-morphological traits include shape, size, morphology and arrangements of the conidia (mitotic spores), vesicle and stipes

(McClenny, 2005; Diba et al., 2007). The presence and morphology of the sexual phase (teleomorph)-associated structures, such as the sclerotia, cleistothecia, Hüll cells and ascospores (meiotic spores) are also important in species-level identification (Klich, 2002). Despite improved and advanced molecular techniques, morphological- and biochemical-based identification of the genus *Aspergillus* is still useful and applicable (Diba et al., 2007).

The genus *Aspergillus* includes species of medical, industrial and scientific importance (Samson & Varga, 2009). The pathogenic species include *Aspergillus fumigatus* (Dagenis & Keller, 2009), *Aspergillus flavus*, *Aspergillus parasiticus* (Hedayati et al., 2007) and *Aspergillus ochraceus* (Bayman et al., 2002). *Aspergillus terreus* is the producer of a cholesterol lowering agent (lovastatin) and itaconic acid (Samson et al., 2011). *Aspergillus oryzae* (koji mould) has been used to ferment the traditional Japanese food and drinks (Murooka & Yamshita, 2008) and *Aspergillus nidulans*/*Emericella nidulans* is the model organism used in gene regulation and molecular biology studies (David et al., 2008).

Thom & Raper (1945) classified the genus *Aspergillus* into several separate groups, and later the genus was divided into six subgenera and eighteen sections (Gams et al., 1985). To date, over 340 *Aspergillus* species have been described (Frisvad, 2015), and among them are species belonging to subgenus *Circumdati* section *Nigri*, i.e. black Aspergilli, which are economically significant. Favourable fermentation behaviour and high levels of protein secretion in addition to being certified as GRAS-organisms make members of the section *Nigri* suitable for industrial and biotechnological applications (de Vries & Visser, 2001; Silva et al., 2011).

*A. niger* was first used in 1919 to produce citric and gluconic acids (Varga et al., 2000). Citric acid is the most important product of *A. niger*, which is used as an acidifier in the food and beverage industries. It is used as a buffer and anti-oxidant in pharmaceuticals and cosmetics, as well as in detergents and leather treating (Roukas, 2000). Furthermore, several secondary metabolites, such as antibiotics, mycotoxins and immunosuppressant agents, are produced by *A. niger*. Among them, ochratoxin A is a contaminant of grains, coffee and grapes (Schuster et al., 2002; Samson et al., 2007).

An advantage of *A. niger* over other *Aspergillus* species is its ability to produce and secrete high levels of extracellular proteins (Schuster et al., 2002). This makes it suitable for use as a protein producing organism for the homologous and heterologous protein production (de Vries et al., 2004; Fleißner & Dersch, 2010). *A. niger* is used as a host for production of a wide spectrum of industrially important hydrolytic enzymes, such as lipases, proteases, amylases and amylo-glucosidases (Abarca et al., 2004; Silva et al., 2011a). In addition, it naturally produces a wide range of plant cell wall polysaccharide-acting enzymes, e.g. cellulases, hemicellulases, pectinases and FAEs (de Vries & Visser, 2001; de Vries, 2003).

## 2. Aims and objectives

The main aim of this thesis was to find and characterize an ascomycete fungal strain that is able to efficiently degrade plant-derived polysaccharides into platform sugars. Hence, several fungal species within the phylum Ascomycota obtained from the Fungal Biotechnology Culture Collection (FBCC) along with other soil-originated isolates, were screened and characterized for their ability to produce plant-polysaccharide degrading enzymes.

The specific objectives were:

1. Screening, isolation, identification and phylogenetic studies of a micro-organism able to depolymerize inulin into monomeric sugars (Publication I).
2. Exploring and comparing the potential of *P. subrubescens* to the industrial enzyme producer, *A. niger* for production of extracellular enzymes for the saccharification of plant biomass (Publication II).
3. Exploring the potential of *A. niger* for the production of accessory enzymes involved in plant biomass degradation (Publication III).



### 3. Materials and Methods

#### 3.1. Fungal strains

Ascomycetous fungi, which were studied in this thesis and in the original publications I, II, and III, are presented in Table 5. Cultivation methods and optimized culture conditions are described in detail in the original publications.

**Table 5.** Fungal species and strains.

Fungal name	Culture collection number	Publication(s)
<i>Aspergillus niger</i>	FBCC <sup>1</sup> 1069	I
<i>Aspergillus niger</i>	FBCC1271	I
<i>Aspergillus niger</i>	FBCC2878	I
<i>Aspergillus niger</i> N402	ATCC64974	II, III
<i>Alternaria infectoria</i> species-group	FBCC1501, 1503, 1505, 1507	I
<i>Alternaria</i> sp.	FBCC1502	I
<i>Alternaria rosae</i>	FBCC1504, 1506	I
<i>Cercospora</i> sp.	FBCC1508	I
<i>Talaromyces rugulosus</i>	FBCC1635	I
<i>Penicillium glabrum</i>	FBCC1637	I
<i>Penicillium raperi</i>	FBCC1636	I
<i>Penicillium subrubescens</i>	FBCC1631, CBS <sup>3</sup> 132782, DTO <sup>3</sup> 188-D5	I
<i>Penicillium subrubescens</i>	FBCC1632, CBS132785 <sup>T</sup> , DTO188-D6	I, II
<i>Penicillium subrubescens</i>	FBCC1633, CBS132784, DTO188-D8	I
<i>Penicillium subrubescens</i>	FBCC1634, CBS132783, DTO 188-D7	I
<i>Penicillium subrubescens</i>	CBS134206, IBT <sup>4</sup> 16650, DTO220-G4	II
<i>Penicillium chrysogenum</i>	CBS307.48	II
<i>Penicillium jamesonlandense</i>	CBS102888	II
<i>Pichia pastoris</i> X-33		III

1. Fungal Biotechnology Culture Collection, University of Helsinki, Finland.

2. Culture collection of Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

3. Internal culture collection of Westerdijk Fungal Biodiversity Institute.

4. Culture collection of Center for Microbial Biotechnology (CBM) at Department of System Biology, Technical University of Denmark.

### 3.2 Plant biomass

The main carbohydrate content of the plant biomasses which were used as substrates for the enzyme detection and enzyme activity measurements besides evaluation of hydrolysis potential of detected enzymes are summarized (Table 6).

**Table 6.** Plant biomass sources.

Plant biomass (manufacturer)	Main polymers content	Pub.
Jerusalem artichoke ground tubers	Inulin	I
Wheat bran (WB) (Wageningen mill, Wageningen, The Netherlands)	Cellulose, hemicellulose	II, III
Sugar beet pulp (SBP) (Weiss BioTech, Ascheberg, Germany)	Cellulose, hemicellulose, pectin	II
SBP pectin (Herbstreith & Fox KG, Neuenbürg, Germany)	Cellulose, hemicellulose, pectin	III

### 3.3. Experimental methods

Experimental set up and the applied methods are described in detail in the original publications (I-III) and summarized (Table 7).

**Table 7.** Experimental methods.

Method	Brief explanation	Publication(s)
Fungal cultivation	Agar plate cultivations	I
	Submerged cultivations	I, II, III
	Bioreactor cultivation	I
Growth profiling	Agar plate cultivations	II
Enzyme activity assays (substrate)	INU (Pure inulin from chicory, Sigma-Aldrich)	I
	INV (Pure sucrose)	I
	EGL (Azo-CM cellulose, S-ACMC, Megazyme)	II
	GAL (Azo-galactan, S-AGALP, Megazyme)	II
	MAN (Azo-carob galactomannan, S-ACGLM, Megazyme)	II
	RHG (AZ-rhamnogalactomannan, S-AZRH, Megazyme)	II

	XLN (Azo-wheat arabinoxylan, S-AWAXP, Megazyme)	II
	ABF ( <i>p</i> NP- $\alpha$ -L-arabinofuranoside, Sigma-Aldrich)	II
	CBH ( <i>p</i> NP- $\beta$ -D-cellobioside, Sigma-Aldrich)	II
	LAC ( <i>p</i> NP- $\beta$ -D-galactopyranoside, Sigma-Aldrich)	II
	BGL ( <i>p</i> NP- $\alpha$ -L-arabinofuranoside, Sigma-Aldrich)	II
	BXL ( <i>p</i> NP- $\beta$ -D-xylopyranoside, Sigma-Aldrich)	II
	FAE (Multiple hydroxycinnamate derived substrates, Apin Chemicals)	III
Protein concentration assays	Bradford protein assay	I
	Modified Lowry protein assay	II
Enzymatic hydrolysis	Fungal culture supernatants	I, II, III
Extracellular protein profiles and molecular mass analysis	SDS-PAGE (12% polyacrylamide gel, Bio-Rad)	I, III
Enzymatic hydrolysis and extrolite products analysis	TLC (Silica gel 60 F <sub>254</sub> Plates, Merck)	I
	HPLC-PAD (Dionex CarboPac PA-1)	I, II
	U-HPLC (Dionex ultimate 3000), HPAEC-PAD HPIC (Dionex ICS-5000 <sup>+</sup> Thermo Scientific).	I III
DNA extraction	Ultraclean <sup>TM</sup> Microbial DNA isolation kit (MoBio)	I
RNA extraction	TRIzol reagent (Invitrogen), RNA isolation kit (NucleoSpin RNA, MACHEREY-NAGEL)	III
cDNA synthesis	ThermoScript Reverse Transcriptase (Invitrogen)	III
Sequence data analysis	MEGA5 software package	I
	Clustal Omega multiple sequence alignment program	III
Gene expression assay	Real time PCR using ABI Fast SYBR Master mix (Applied Biosystems)	III
Cloning	pPICZ $\alpha$ A (Invitrogen)	III
Media		
Malt extract agar	MEA	I

Blakeslee Malt extract agar	Blakeslee MEA	I
Czapek yeast autolysate agar	CYA	I
CYA supplemented with 5% NaCl	CYAS	I
Yeast extract sucrose agar	YES	I
Creatine agar	CREA	I
Dichloran 18% glycerol agar	DG18	I
Oatmeal agar	OA	I

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## 4. Results and discussion

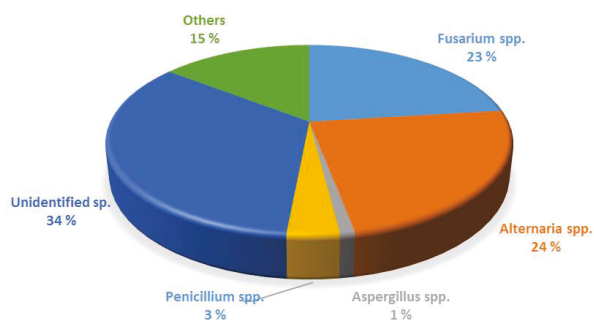
### 4.1. Isolation, screening and description of native fungi able to degrade the polyfructan inulin (I)

#### 4.1.1. Isolation and agar-plate screening of fungi

To find natural degraders of the polyfructan inulin, which constitutes much of the reserve biomass of flowering plants, 126 fungal strains of various species within the phylum Ascomycota (the majority of which were *Aspergillus*, *Alternaria*, and *Fusarium* species) from the FBCC and 74 strains isolated from plantation soil of flowering plants were screened for inulin degradation on agar plates (Fig 14). Fungal growth rates and generation of clear halo zones around the grown colonies on the agar plates (Li et al., 2011) were two main criteria that were considered indicative of inulinase production (Figs. 15 A & B).

Apart from the *A. niger* strains that were used as the positive controls, other fungal strains obtained from the FBCC showed weak to moderate growth on agar plates without observable halo zones. Of the 74 strains isolated in Finland from soil around Jerusalem artichoke (*Helianthus tuberosus*) tubers, seven isolates (FBCC1635, FBCC1637, FBCC1631, FBCC1632, FBCC1634, FBCC1633, FBCC1636) grew well on agar plates amended with inulin and Jerusalem artichoke as the carbon sources. As an indication of inulin-degrading activity, three isolates (FBCC1631, FBCC1632 and FBCC1634) formed

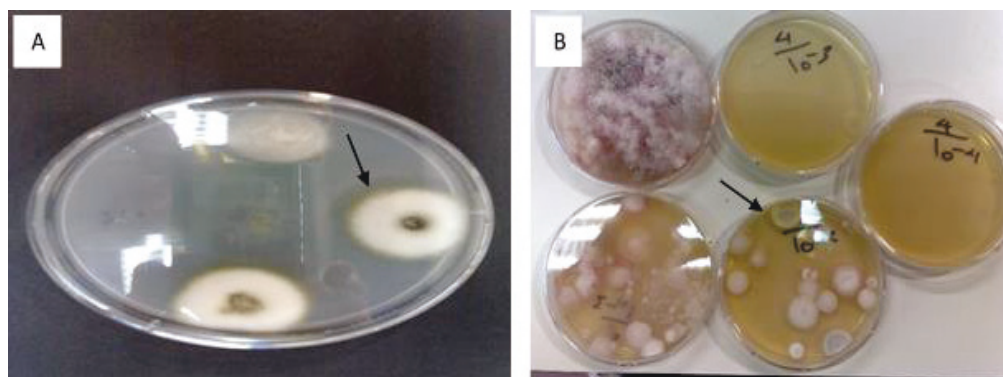
observable clear zones on inulin-supplemented agar plates (Fig. 15B). All these strains were preliminarily identified as *Penicillium* sp. using light microscopy. The other 67 isolates showed no remarkable growth and did not produce clear zones on the agar plates. Two *A. niger* strains (FBCC1271 and FBCC2878), which were used as positive controls, showed favourable growth and formed colonies with clear zones on inulin-supplemented plates, indicating their ability to hydrolyse inulin (Fig. 15A).



**Figure 14.** A total number of 200 fungal strains were screened on agar and in liquid culture media to find efficient inulin degraders. These included 46 *Alternaria* strains, 48 *Fusarium* strains, 2 *Aspergillus* strains, and 74 soil-originated isolates of which 67 isolates were left unidentified. Others represent 30 strains belonging to the species such as *Cladosporium* sp., *Epicoccum* sp., *Cercospora* sp., *Mucor* sp., *Phoma* sp., *Sordaria* sp., *Aurobasidium* sp. and *Torula* sp.

An important factor that influences inulinase production is the composition of the culture medium (Rawat et al., 2015a). Inulin and inulin-containing plant biomass are able to induce the production of exo- and endo-type inulinase activities (Galindo-Leva et al., 2016). Therefore, pure inulin from dahlia tuber (Sigma-Aldrich) and Jerusalem artichoke tuber preparations were used as the sole sources of carbon in this study. Pure inulin induces high levels of inulinase

production by *Penicillium* sp. TN-88, compared with other carbon sources (Nakamura et al., 1997). Similarly, pure inulin in this study was the best inducer for inulinase production for the inulinase-positive strains in liquid culture media. However, to decrease the cost of enzyme production, natural inulin-containing biomass was recommended for as a favourable inducer (Kango et al., 2003).



**Figure 15.** A) *A. niger* strain FBCC2878 cultivated on the pure inulin containing medium. B) Strain FBCC1632 isolated from plantation field of flowering plants containing pure inulin as sole source of carbon. Clear halo zones around fungal mycelia are indicated with arrows.

#### 4.1.2. Screening of inulinase activity in submerged cultures

In addition to screening on solid culture media, the set of 200 fungal strains from the FBCC and fresh isolates were further screened in liquid culture media supplemented with pure inulin from dahlia tubers and a Jerusalem artichoke preparation. The hydrolytic activity of crude culture filtrates towards inulin (I) or sucrose (S) was measured to calculate the I/S ratio, which represents the ratio of the activity of the enzyme preparations on inulin and sucrose. It was used to define the substrate preference of the crude enzyme and to predict endo- and

exo-type activities (Nakamura et al., 1978; Vandamme & Derycke, 1983). An I/S ratio of high values shows that inulinase is predominant, compared with the invertase (SUC) activity of which the enzyme is more active on the glycosidic bonds within the inulin chain (endo-type activity), while the contrasting trend is considered to predict exo-type inulinase activity. In all, 17 out of 200 fungal strains of various genera showed detectable inulinase activity. Exo- or endo-type activities were predicted, based on the SUC activities produced. Apart from the inulinase-positive strains, other strains produced no remarkable cell mass in the liquid culture media, and detectable SUC activities were not observed.

A total of 5 out of 7 *Penicillium* species favoured pure inulin as a substrate. Apart from *Penicillium glabrum* FBCC1637 (see 4.1.4 and 4.1.5), which showed remarkable inulinase compared with SUC activity, indicating endo-type activity (I/S ratio of 6.7), the other strains were interpreted as producers of exo-type inulinase activity. Freshly isolated *Penicillium* sp. FBCC1632, which subsequently was identified as a new *Penicillium* species named *Penicillium subrubescens* (see 4.1.4 and 4.1.5), was the most efficient inulinase producer in submerged cultivation amended with pure inulin (Fig. 16). It showed inulinase and SUC activities of 7.7 and 6.1 U ml<sup>-1</sup>, respectively, with an I/S ratio of 1.3, which indicated exo-type hydrolytic activity. Previously, exo-acting inulinases were reported from *P. trzebinskii* (crude enzyme) and *P. janczewskii* (purified enzyme) with I/S ratios of 2.6 and 1.2, respectively (Onodera & Shiomi 1992; Pessoni et al., 1999; Pessoni, 2007). In addition, Nakamura et al. (1997) showed that of various carbon sources, pure inulin induced a maximum inulinase production of up to 9.5 U ml<sup>-1</sup> after 4 days of cultivation by *Penicillium* sp. TN-88. I/S ratios of 8.7 and 50 were also reported for crude INU produced by

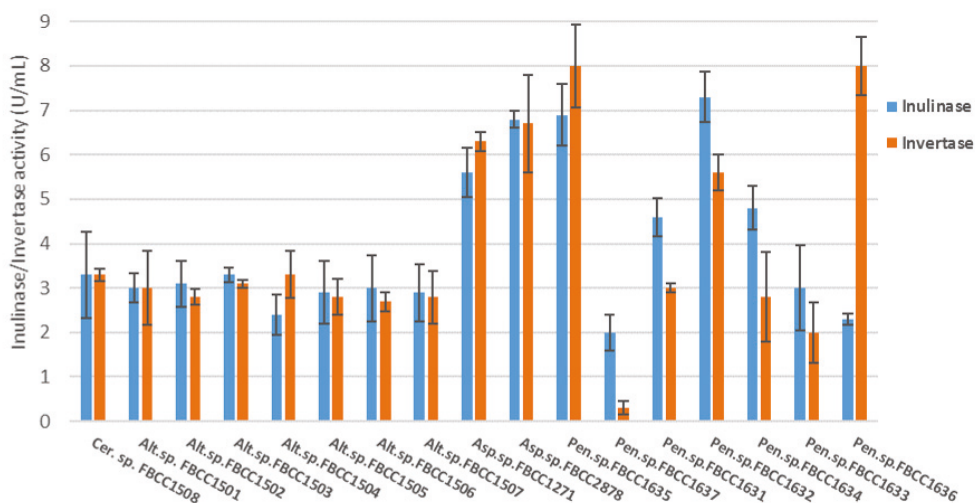
*Penicillium* sp. TN-88 and a purified INU of *P. purpurogenum*, respectively (Onodera & Shiomi 1988; Nakamura et al., 1997).

The genera *Penicillium*, *Aspergillus*, *Alternaria* and *Fusarium* are among the most well-known fungi capable of producing inulinases (Abd Al-Aziz et al., 2012). *Aspergillus* species from section *Nigri* are known as prominent inulinase producers (Pandey et al., 1999; Kango 2008). Under optimized cultivation conditions, up to 11.3 U ml<sup>-1</sup> inulinase activities have been detected from *Aspergillus* species of section *Nigri* (Rawat et al., 2015a).

The culture filtrates of the positive control strains *A. niger* FBCC1271 and FBCC2878 showed favourable hydrolytic activities towards inulin and sucrose with the highest inulinase activities of 5.6 and 6.8 U ml<sup>-1</sup> and SUC activities of 6.3 and 6.7 U ml<sup>-1</sup>, respectively (Fig. 16). The I/S ratios of 0.9 and 1.0 indicated equal preference for both inulin and sucrose, respectively. *Alternaria* spp. FBCC1501, FBCC1502, FBCC1503, FBCC1505, FBCC1506 and FBCC1507 showed low inulinase activity, which was similar to the SUC activity (I/S ratio app. 1.0). The only exception was *Alternaria* sp. FBCC1504, which showed higher SUC than inulinase activity (I/S ratio app. 0.7).

No inulinase activity was observed in the culture filtrates of *Fusarium* species. Several studies have shown inulinase production in *Alternaria* and *Fusarium* species, of which *Alternaria alternata* (Hamdy 2002; Sanal et al., 2005) and *Fusarium oxysporum* (Gupta et al., 1992) are among the well-studied species.

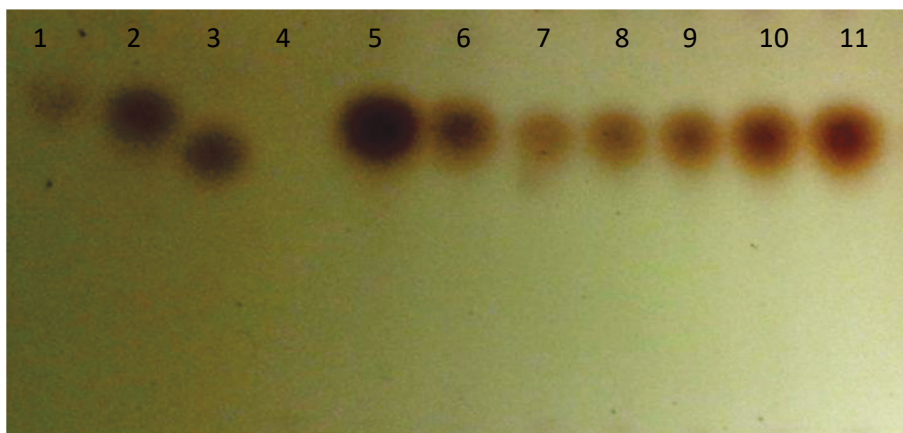




**Figure 16.** Inulinase and invertase activities measured from 5 days old culture filtrates of Alt: *Alternaria* sp., Asp: *Aspergillus* sp., Pen: *Penicillium* sp. and Cer: *Cercospora* sp. Error bars represent standard deviation between three replicates.

#### 4.1.3. Saccharification of inulin by *P. subrubescens* enzymes and analysis of hydrolysis products

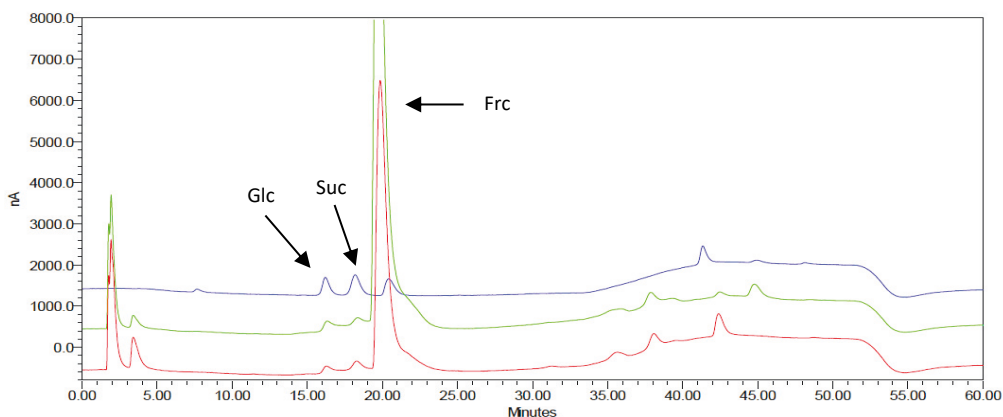
The crude enzyme mixture obtained from day 4 bioreactor cultivation of *P. subrubescens* FBCC1632 was used for saccharification of pure inulin isolated from dahlia tubers. The end products of saccharification were analysed by thin layer chromatography (TLC) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). TLC analysis after hydrolysis of inulin illustrated intense spots that corresponded both to the Frc standard and the commercial inulinase reaction end-products (Fig. 17).



**Figure 17.** TLC chromatogram of inulin hydrolyzed by *P. subrubescens*. Lanes 1–4: standards. Lane 1: Glc, lane 2: Frc, line 3: sucrose and lane 4: pure inulin. Lanes 5 and 6 are hydrolysis products of inulin where 5 and 2.5 U ml<sup>-1</sup> commercial exo-inulinase were used, respectively. Lanes 7–11 are end-products after 24 hours of hydrolysis of inulin in the presence of crude enzyme mixture of *P. subrubescens* FBCC1632 (1, 2, 3, 4 and 5 µl in lanes 7-11, respectively). Reprinted from publication I with permission from Springer.

The main end-product of exo- acting inulinase is Frc, while endo-acting enzymes release oligofructans of DPs of F3, F4 and F5 (Onodera & Shiomi, 1988). The absence of fructooligosaccharides and the release of Frc after hydrolysis showed that *P. subrubescens* was able to demonstrate exo-type inulinase activity that hydrolysed the glycosidic bonds in inulin and converted it into Frc (Fig. 17). This was also verified by HPAEC-PAD analysis (Fig. 18).

Previously, TLC analysis has been used to show the production of exo- and endo-acting inulinases by *Penicillium spinulosum* and *P. purpureogenum*, respectively (Onodera & Shiomi, 1988; Ertan et al., 2003). Furthermore, exo- and endo-inulinase mixtures produced by *Penicillium* sp. NFCC (National Fungal Culture Collection) 2768 release both Frc and oligofructans from chicory inulin in TLC analysis (Rawat et al., 2015).



**Figure 18.** HPAEC-PAD chromatogram of the hydrolysis products of inulin using *P. subrubescens* FBCC1632 culture filtrate (red) compared to hydrolysates of inulin saccharification by commercial inulinase of *A. niger* (green). Frc was the main monosaccharide released after 24 h hydrolysis and no oligofructans were detected. Standard (blue), Glc: glucose, Suc: sucrose, Frc: fructose.

#### 4.1.4. Morphological identification of the inulinase-positive strains and extrolite production

The macro- and micro-morphological characteristics of *P. subrubescens* FBCC1632 were analysed and compared with the representative *Penicillium* species within section *Lanata-Divaricata*. *Penicillium ochrochloron*, *Penicillium piscarium*, *Penicillium pulvillorum*, *Penicillium rolfsii*, and *Penicillium svalbardense* represent features common to *P. subrubescens*, such as rapid growth at 25°C, formation of a distinct neck, rough-walled and predominantly biverticillate conidiophores, as well as absence of ascomata and sclerotia, which is typical for the sexual stage. *P. subrubescens* developed a pinkish red reverse colour on yeast extract sucrose (YES) agar medium, according to which the species was named. The general phenotypic data obtained from 7-days-old cultures of *P. subrubescens* FBCC1632 on Czapek

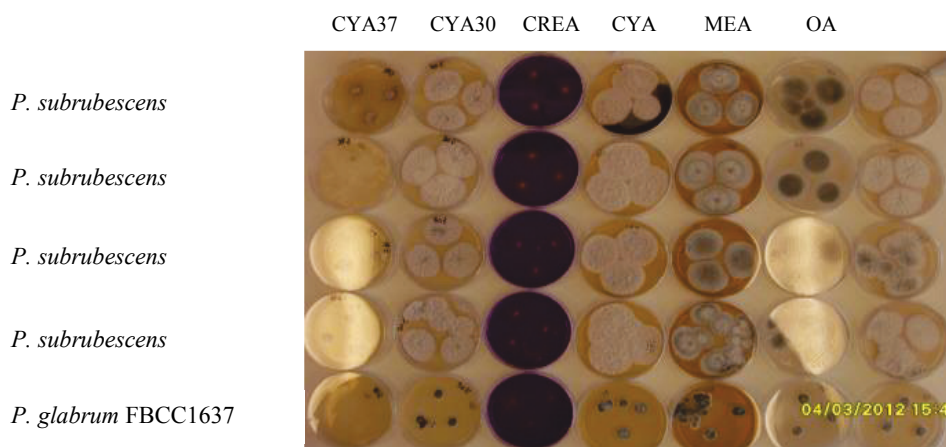
yeast autolysate (CYA) agar, CYA at 30 °C, YES, oatmeal agar (OA), and malt extract agar (MEA; Oxoid Company, ThermoFisher Scientific Waltham, MA, USA) at 25 °C are summarized in Table 9.

**Table 9.** Morphological characteristics of *P. subrubescens* FBCC1632 from 7-days-old cultivations on selected media.

Medium*	Colony shape	Colony size (diam, mm)	Mycelium color	Conidiogenesis	Exudate & soluble pigment	Reverse color
CYA	Spreading, low, slightly raised in center, radial, entire	40-50	White to pale beige	Absent	Absent	Beige to light brown
CYA 30°C	Spreading, low, slightly raised in center, radial, entire	40-55	White to pale beige	Absent	Absent	Beige to light brown
YES	Spreading, low, wrinkled sulcate	45-60	White	Absent	Absent	Pink
MEA	Floccose	40-55	White	Light to moderate dens; conidia <i>en mass</i> grey-green	Clear minute droplets occasionally produced	Unaffected with yellow-brown center
OA	Spreading	40-45	White	Moderate dens; conidia <i>en mass</i> grey-green	Absent	Light brown

\*Malt extract agar (MEA), Czapek yeast autolysate (CYA) agar, yeast extract sucrose (YES) agar, creatine agar (CREA), oatmeal agar (OA).

Two unique features were used to distinguish *P. subrubescens* from other phenotypically similar species. The *P. subrubescens* strains grew weakly on creatine agar (CREA) and restrictedly on CYA at 37°C. With the exception of *P. glabrum* FBCC1637, the colony characters of the four inulinase producers represent the strains belonging to the novel *Penicillium* species *P. subrubescens* sp. nov. (Fig. 19).



**Figure 19.** Four *P. subrubescens* isolates (FBCC1632, FBCC1633, FBCC1634, and FBCC1631) besides *P. glabrum* FBCC1637 cultivations isolated from plantation field soil of Jerusalem artichoke. Cultivations on MEA, OA, YES, CREA, and CYA were grown at 25°C and CYA at 30°C and 37°C.

The weak growth of *P. subrubescens* on CREA was similar to that of *P. ochrochloron*, *P. piscarium*, *P. pulvillorum* and *P. rolfsii* while *P. svalbardense* grew moderately well on this medium. Furthermore, the very restricted growth of *P. subrubescens* on CYA at 37 °C was different from that of the other section representatives, which either did not grow at all or grew rapidly, forming large colonies. Together with micro-morphological characters, such as conidia ornamentations, which were globose, subglobose or broadly ellipsoidal, macro-morphological characters indicated that *P. subrubescens* was most similar to *P. pulvillorum*.

The most notable variation was that the *P. subrubescens* strain developed a pinkish red reverse colour on YES and Blakeslee MEA caused by an uncharacterized extrolite tentatively named ‘red’ or ‘verni’. *P. subrubescens* was also able to produce a unique combination of extrolites, such as andrastin A, penicillic acid, pulvilloric acid, xanthoepocin and janthirens. Based on

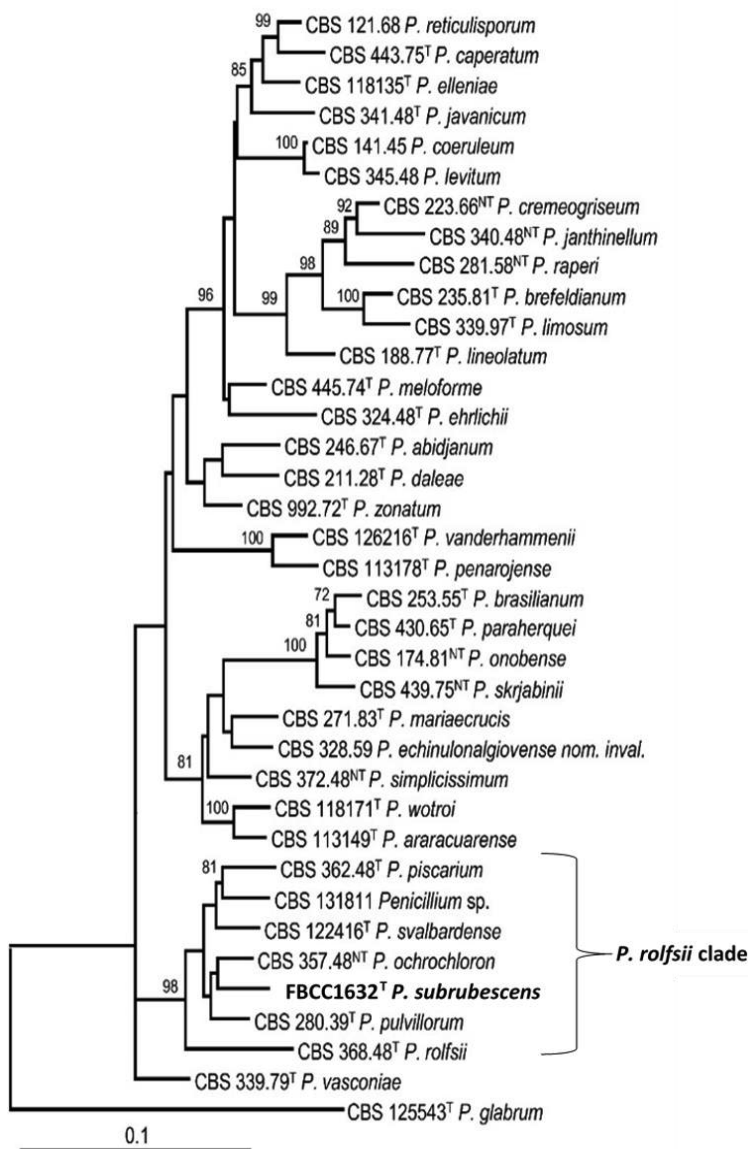
these findings and phylogenetic analysis, *P. subrubescens* FBCC1632 was named as *P. subrubescens* sp. nov., which represents a novel species in section *Lanata-Divaricata* in subgenus *Aspergilloides*. Etymologically, ‘subrubescens’ means ‘becoming pink’, referring to the developing pinkish red reverse colour on YES and Blakeslee MEA.

Detailed growth profiles, colony characters and exudate production analysis of *P. subrubescens* FBCC1632 were carried out recently by Visagie et al. (2015). The unique features of *P. subrubescens*, which have been compared with other members of section *Lanata-Divaricata* in subgenus *Aspergilloides* support our findings. Section *Lanata-Divaricata* was introduced by Thom (1930) describing species with biverticillate conidiophores and *Penicillium janthinellum* as the type species. The section taxonomy was rebuilt by Houbraken and Samson (2011), based on combined data obtained from ITS and BenA sequences of 38 fungal species. Recently 43 species were described as belonging to this section (Visagie et al., 2015). Members of the section *Lanata-Divaricata* commonly originate from the surface soil of local plant communities, particularly in grasslands (Christensen et al., 2000) and leaf litter (Houbraken et al., 2011b), which is in line with the agricultural plantation field soil origin of *P. subrubescens* FBCC1632.

#### **4.1.5. Taxonomy and classification**

Molecular characteristics, e.g. ITS, calmodulin (*cmd*),  $\beta$ -tubulin (*benA*) and RNA polymerase II (*RPB2*) gene sequences, were analysed for taxonomic classification and investigation of the phylogenetic relationships of *P. subrubescens* with other members of *Penicillium* section *Lanata-Divaricata* (Publication I). Phylogenetic analysis showed that *P. subrubescens* belongs to the *P. rolfsii* clade, which is strongly supported with a separate evolutionary

descent (Fig. 20). The clade *P. rolfsii* comprises *P. pulvillorum*, *Penicillium cieglerei*, *P. svalbardense*, *P. piscarium*, *P. ochrochloron* (Houbraken et al., 2011b) and the recently described species *Penicillium annulatum* (Visagie et al., 2015). Combined results obtained from phenotypic examination together with multigene and phylogenetic analysis proved that *P. subrubescens* represents a new species within section *Lanata-Divaricata*, subgenus *Aspergilloides*.



**Figure 20.** Best-scoring maximum likelihood tree using RAxML based on a combined data set of partial  $\beta$ -tubulin and ITS sequences of *P. subrubescens* FBCC1632 (CBS 132785). Well-supported branches (> 95% bootstrap supported) are in bold. *Penicillium glabrum* CBS 125543<sup>T</sup> was used as an outgroup. Modified from publication I with permission from Springer.



## **4.2. Plant biomass-degrading potential of *P. subrubescens* (II)**

### **4.2.1. Growth profiles predict the potential for plant biomass degradation**

The growth profiles of four *Penicillium* strains, *P. subrubescens* FBCC1632, *P. subrubescens* CBS 134206 (Westerdijk Fungal Biodiversity Institute, Filamentous Fungi and Yeast Collection) (data not shown), *Penicillium chrysogenum* CBS 307.48 and *Penicillium jamesonlandense* CBS 102888, and *A. niger* N402 were performed on minimal medium (MM) agar plates supplemented with mono-, oligo-, and polysaccharides, as well as a set of complex plant-derived biomasses (Fig. 21). The ability of growth and colony formation indicated that the strains examined were able to utilize the available carbon sources and metabolize the monomeric sugars released.

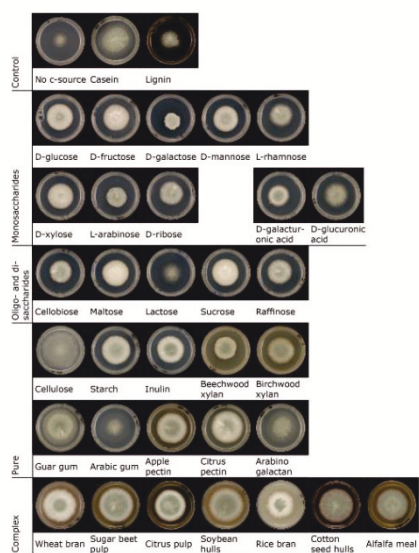
The growth of the *P. subrubescens* strains was superior to that of the other two *Penicillia* but similar to that of *A. niger* N402. However, the *P. subrubescens* strains were able to degrade or metabolize two monomeric sugars (D-Gal, D-ribose) and two polysaccharides (Arabic gum and arabinogalactan) more efficiently than *A. niger*. They were also able to grow on calcium lignin, which is a low-carbohydrate lignin preparation, thus indicating their higher tolerance towards lignin than *A. niger*. In addition, the weak growth of the *P. subrubescens* strains on casein suggested low protease production, which may indicate the ability to secrete a wide range of proteins. Both *P. subrubescens* strains and *A. niger* grew well on the low-lignin-containing plant biomasses, i.e. wheat bran (WB) and sugar beet pulp (SBP), and a crude lignocellulose feedstock of cottonseed pulp (Fig. 21). The structural composition of WB and SBP are significantly different (Ozturkoglu Budak et al., 2014). Due to the notable growth of both *Penicillia* and *A. niger* on WB and SBP, they were selected as carbon sources in the subsequent comparative experiments. The

main compositions of the plant biomass substrates used in this study are summarized in Table 10. Considering the structural composition of WB and SBP and for complete decomposition, cellulase, hemi-cellulase and pectinase activities would be needed.

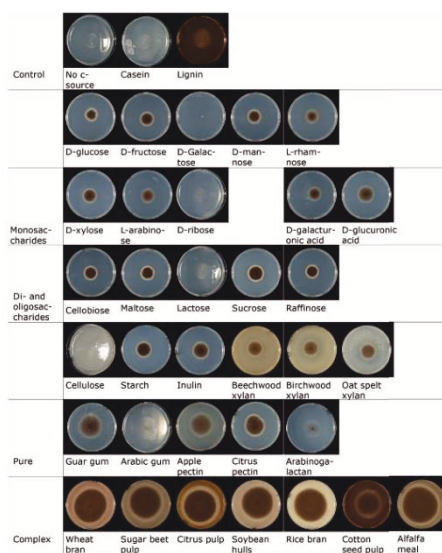
**Table 10.** Monosaccharide composition (mass-%) and the corresponding polysaccharides of the plant biomasses used as growth substrates.

	Rha	Ara	Xyl	Man	Gal	Glc	Ura	Polysaccharides
Wheat bran	0	17	35	1	2	42	3	Major components cellulose and glucuronoarabinoxylan
Sugar beet pulp	1	28	2	2	7	33	26	Major components cellulose, pectin and xyloglucan
Birch wood xylan			85.6				8.7	

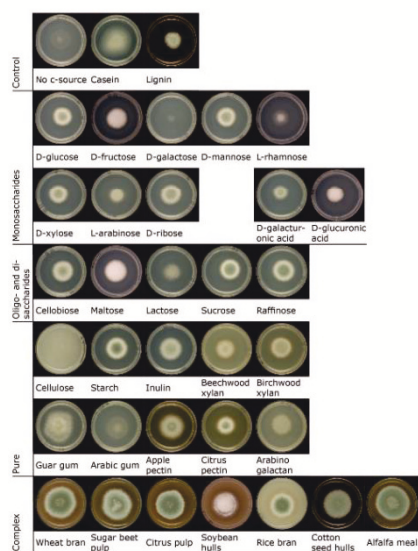
Rha: Rhamnose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, Ura: uronic acids (modified from Ebringerova, 2005; Ozturkoglu Budak et al., 2014).



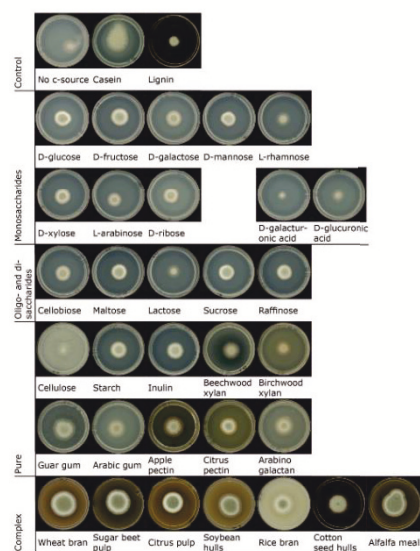
***Penicillium subrubescens* FBCC1632**



***Aspergillus niger* N402**



***Penicillium chrysogenum* CBS307.48**



***Penicillium jamesonlandense* CBS102888**

**Figure 21.** Growth profiles of *P. subrubescens* FBCC1632, *A. niger* N402, *P. chrysogenum* and *P. jamesonlandense*, on a set of different mono-, oligo-, and polysaccharides and complex plant-derived biomasses.

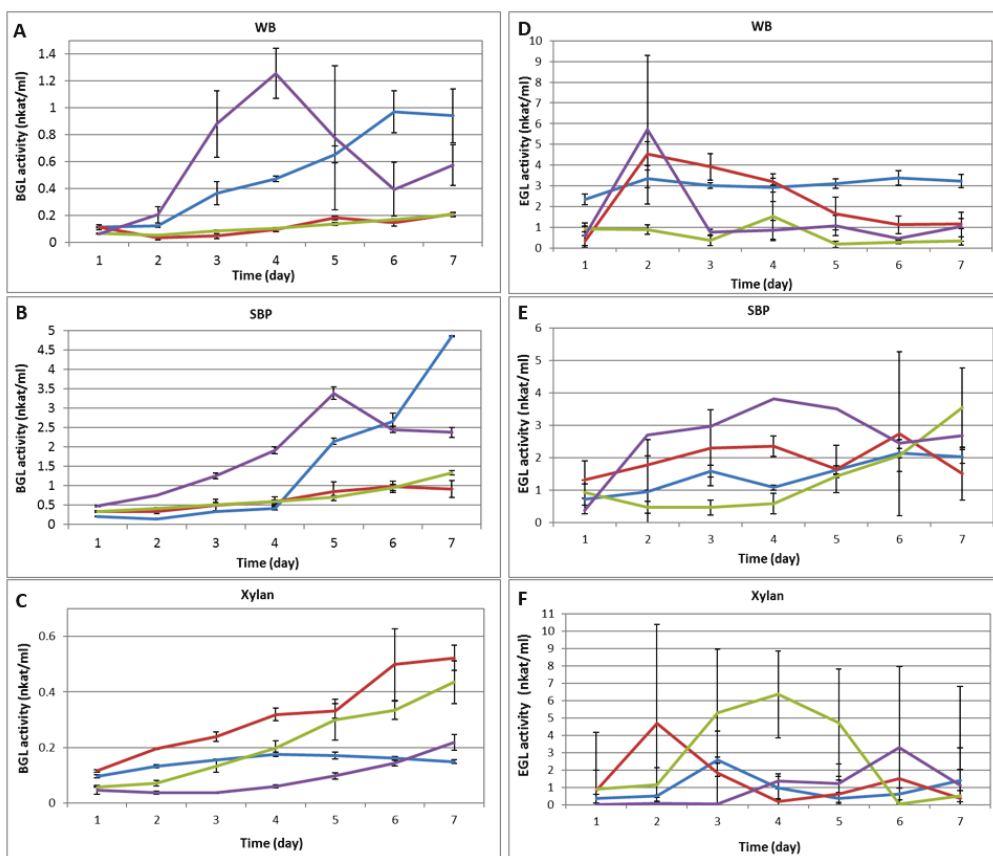
#### 4.2.2. Cellulolytic and hemicellulolytic potentials of selected *Penicillia*

*Penicillia* were grown in MM supplemented with WB, SBP or birch wood xylan and their cellulolytic and hemicellulolytic potentials were evaluated by measuring endo-glucanase (EGL), cellobiohydrolase (CBH), and  $\beta$ -glucosidase (BGL), and xylanase (XLN) activities in the culture supernatant (Figs. 22 & 23).

All *Penicillia* showed relatively low BGL and EGL activities, despite favourable growth observed on cellulose plates (Fig. 22). The highest BGL activity, 4.9 nkat ml<sup>-1</sup>, was obtained after 6 days of cultivation on SBP by *P. subrubescens* FBCC1632 (Fig. 22B), while cultivation on WB and xylan resulted in similar activity levels in all *Penicillia* cultivations (Fig. 22A,C). The highest EGL activity (6.4 nkat ml<sup>-1</sup>) was detected in birchwood xylan-supplemented cultivations of *P. chrysogenum* on day 4 (Fig. 22F).

Comparison of the previously reported cellulase activity levels is difficult due to the diversity of the culture and assay conditions applied. However, among the well-studied *Penicillia*, *Penicillium decumbens* has been used for the production of cellulases on an industrial scale (Fang et al., 2010). WB was used for BGL induction in *P. decumbens* 114-2 and *P. decumbens* JU-A10, in which 2.38 U ml<sup>-1</sup> and 1.02 U ml<sup>-1</sup> activities were achieved, respectively (Sun et al., 2008).

High BGL and EGL activities were reported in 4-day-old cultivations of *Penicillium echinulatum* 9A02S1 grown on different combinations of WB and sugar cane bagasse (Camassola & Dillon, 2007). In addition, activity of 5.5 U ml<sup>-1</sup> was reported when WB was used to induce BGL production by a mutant strain of *Penicillium pinophilum* (NTG III/6) (Brown et al., 1987).



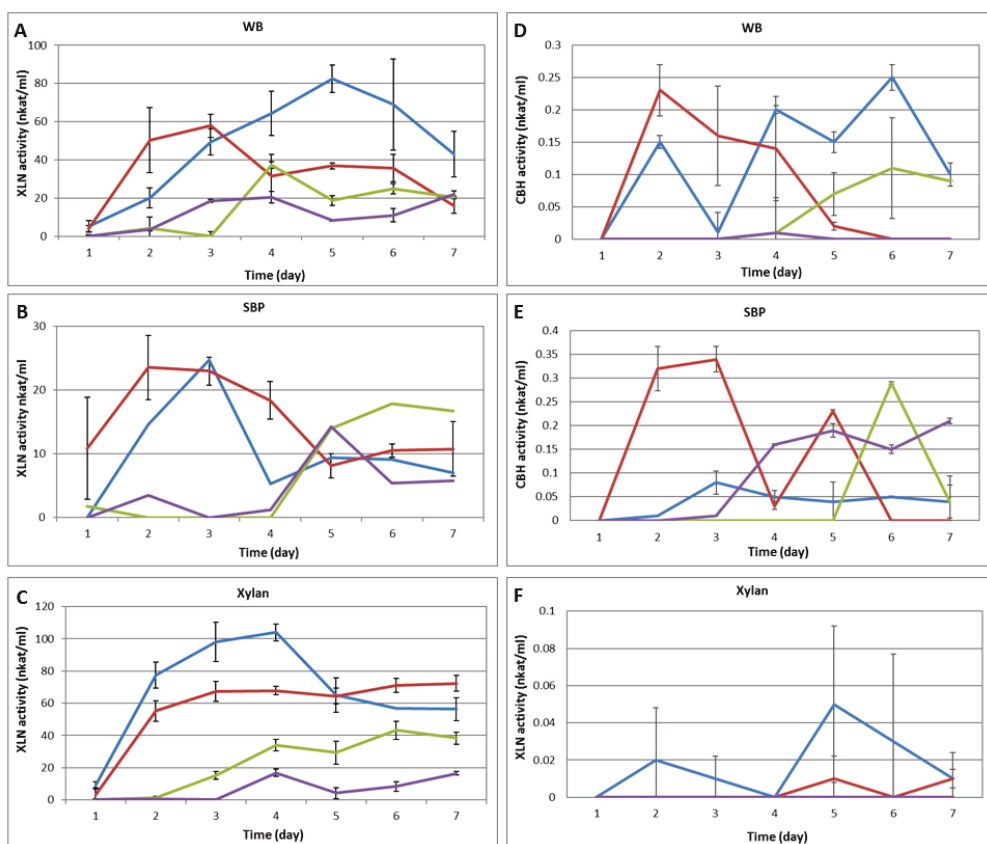
**Figure 22.** BGL activity on on A) WB, B) SBP and C) birch wood xylan cultivations, and EGL activity on D) WB, E) SBP and F) birch wood xylan cultivations. *P. subrubescens* FBCC1632 (blue), *P. subrubescens* CBS134206 (red), *P. chrysogenum* (green), and *P. jamesonlandense* (purple). The error bars represent standard deviation between three replicates.

A combination of birchwood xylan, oat spelt xylan and Sigmacell type 20 cellulose resulted in  $1.09 \text{ U ml}^{-1}$  and  $2.59 \text{ U ml}^{-1}$  activities of EGL and BGL, respectively, by *Penicillium brasilianum* IBT2088 (IBT Cultural Collection of Fungi, DTU Systems Biology, Technical University of Denmark) (Jørgensen & Olsson, 2006). By converting  $\text{U ml}^{-1}$  to  $\text{nkat mL}^{-1}$  ( $1 \text{ U ml}^{-1} = 16.65 \text{ nkat mL}^{-1}$ ), we concluded that *P. subrubescens* showed lower cellulase activities under

the cultivation condition studied than the well-known cellulase producing *Penicillia*. However, it was speculated that optimization of the culture conditions might result in higher activities.

Very low CBH activities were produced by the *Penicillium* strains examined (Fig. 23). The highest CBH activity ( $0.3 \text{ nkat ml}^{-1}$ ) was produced by *P. subrubescens* CBS134206 on day 3 in the medium supplemented with SBP (Fig. 23E). The highest XLN activity ( $103.9 \text{ nkat ml}^{-1}$ ) was detected in birchwood xylan-supplemented cultivations by *P. subrubescens* FBCC1632 (Fig. 23C). Furthermore, considering the varying content of xylan polysaccharides in WB (mainly glucuronoarabinoxylan) and SBP (mainly xyloglucan) (Table 10), the former induced the highest XLN activity by *P. subrubescens* FBCC1632 (Fig. 23A), while among the *Penicillia* tested relatively low XLN activities were detected in SBP-supplemented cultivations (Fig. 23B).

In line with the current study, pure xylan induced higher XLN activity than WB in *P. glabrum* (Knob et al., 2013) and *P. janczewskii* (Terrasan et al., 2010). However, WB was a more effective XLN inducer ( $6.47 \text{ U ml}^{-1}$ ) for *P. chrysogenum* PCL501 isolated from wastewood than pure xylan, sugarcane pulp and sawdust (Okafor et al., 2007). Moreover, WB was superior to sugarcane bagasse, rice straw and rice bran in inducing CBH activity by *P. funiculosum* NCL1 under submerged and solid-state fermentation (Vanitha et al., 2014).



**Figure 23.** XLN activities on A) WB, B) SBP and C) birch wood xylan, and CBH activities on D) WB, E) SBP and F) birch wood xylan supplemented cultivations. *P. subrubescens* FBCC1632 (blue), *P. subrubescens* CBS134206 (red), *P. chrysogenum* (green), and *P. jamesonlandense* (purple). The error bars represent standard deviation of three replicates.

These results convey that *P. subrubescens* FBCC1632 was able to produce (hemi)cellulose-degrading enzyme cocktails superior to those of the other *Penicillia* examined. Furthermore, the results showed that the hydrolytic activities of the *Penicillia* selected are significantly affected by the substrate composition. This phenomenon was clearly observed while the high-xylan polysaccharides content of WB led to high levels of XLN production

comparable to that of pure xylan by *P. subrubescens* FBCC1632. Consistent with the current study, WB was the best inducer for XLN activity by *Penicillium sclerotiorum* while the highest specific activities were detected in the presence of xylan (Knob & Carmona, 2008). XLN induction by WB was reported in *P. expansum*, *Penicillium oxalicum* ZH-30 and *P. chrysogenum* PCL501 (de Souza Querido et al., 2006; Li et al., 2007; Okafor et al., 2007). SBP, which is a by-product of the sugar-refining industry, contains 22-30% of both cellulose and hemicellulose (mainly containing arabinose), 24-32% of dry matter pectin and a low lignin content (less than 6%) (Table 10). It has mainly been used as a substrate for the production of pectinolytic rather than cellulolytic enzymes (Azzaz et al., 2013; Heerd et al., 2014; Tepe & Dursun, 2014).

In addition to low lignin content (1-3%), WB has high protein (up to 19%) and high cellulose and hemicellulose contents (more than 30% each), which makes it a good inducer of cellulases and hemicellulases (Falkoski et al. 2013). The high protein content of WB negatively influences the production of cellulases and XLN, while its soluble cellooligosaccharides positively affect the production of cellulases (Sun et al., 2008). The low induction effect of WB in the current study may have been due to the high protein and moisture contents of WB or loss of its activities in the solid fraction while the crude culture filtrate was extracted. Production of diverse sets of enzyme activities while switching from one substrate to another confirmed that ascomycete fungi employ diverse approaches for plant biomass degradation (Benoit et al., 2015).



#### 4.2.3. Plant biomass degrading ability of *P. subrubescens* FBCC1632 and *A. niger* N402

The potential of *P. subrubescens* FBCC1632 to degrade complex plant-derived biomass was compared with that of *A. niger* N402 in liquid culture media supplemented with WB (1% w/v), SBP (1% w/v) and an equal combination of these (0.5% w/v each). The samples were collected after 3 and 6 days of cultivation to evaluate their cellulolytic, hemicellulolytic and pectinolytic activities.

*P. subrubescens* showed higher EGL activity (39.77 times the amount of dye released/mg of total protein) in SBP-supplemented cultivations than did *A. niger* (17.25 times the amount of dye released/mg of total protein) after 3 days of cultivation. Furthermore, the high pectinolytic activities were produced by both *P. subrubescens* and *A. niger*. Considerable amounts of endo- $\beta$ -1,4-galactanase (GAL),  $\alpha$ -rhamnosidase (RHA),  $\beta$ -galactosidase (LAC) and  $\alpha$ -arabinofuranosidase (ABF) were produced, especially in those cultivations that were supplemented with SBP, which has a high pectin content. Both *P. subrubescens* and *A. niger* produced endo-xylanases (XLNs) and mainly  $\beta$ -xylosidase (BXL) in the medium supplemented with WB. The results were consistent with the fact that the main hemicellulose of WB is glucuronoarabinoxylan, while SBP contains mainly xyloglucan (Ozturkoglu Budak et al., 2014). In WB-amended cultivations, the XLN activity of *P. subrubescens* (31.66 times the amount of dye released/mg of total protein) was two times higher than that of *A. niger* (15.01 times the amount of dye released/mg of total protein), while BXL activities were also detected. This is in line with previous studies on several other *Penicillia* that have reported to produce xylanolytic enzymes with remarkable attributes for biotechnological purposes (Polizeli et al., 2005; Chavez et al., 2006; Ritter et al., 2013; Liao et

al., 2015). In addition, a recent study showed that WB induced xylanolytic activities (XLN and BXL) in *A. niger* (Benoit et al., 2015).

In addition to a broad range of similar plant polysaccharide-degrading enzymes produced by both species, an interesting correlation between substrate composition and production of well-tuned enzyme cocktails was observed. Low endo-mannanase (MAN) activities (ranging from 0.20 to 11.53 times the amount of dye released/mg of total protein) were detected, which correlates with the low amount of Man in WB and SBP (Ozturkoglu Budak et al., 2014). In addition, the high pectin content of SBP (Ozturkoglu Budak et al., 2014) induced notably higher activities of GAL, LAC and ABF in SBP and in the cultivations supplemented with the mixture of WB and SBP. These results illustrate favourable matching between substrate composition and enzyme production. The results also indicated that the mixture of WB and SBP reinforces the enzymatic activities, compared with individual substrates. These phenomena, which were observed both for MAN and EGL activities, especially in *P. subrubescens*, suggested a more efficient induction of (hemi-)cellulase production in the mixed substrate. Consistent with the current study, we found that the cellulase and hemicellulase induction systems of *T. reesei* RUT C-30 are inducible by WB and SBP (Olsson et al., 2003; Singhania et al., 2007). Higher levels of cellulase and hemicellulase were also produced when the induction system of *T. reesei* RUT C-30 induced by a carbon source that is a mixture of two different substrates (Olsson et al., 2003).

The enzyme production profile of *P. subrubescens* indicates that it is able to depolymerize complex plant biomass by producing a broad range of hydrolytic enzymes. This is in line with previous reports in which the ability of *Penicillia* to produce plant cell wall polysaccharide-degrading enzymes was investigated (Chaabouni et al., 1994; Krogh et al., 2004; Jørgensen et al., 2005). A similar

set, but different ratio, of plant polysaccharide-degrading enzymes was produced by both *P. subrubescens* and *A. niger*, suggesting that different regulatory systems control the enzyme production. In the genus *Aspergillus*, a number of transcriptional regulators that control the genes encoding carbohydrate-active enzymes have been reported. The mechanism of the regulation is based on the switching of the expression of the target genes on and off, which leads to control of plant biomass consumption (Kowalczyk et al., 2014). In *Penicillia* the molecular mechanisms that control the regulation of plant biomass consumption are poorly understood, and the regulation is studied mostly based on the induction effects of the carbon sources consumed and the repression effects of the hydrolysis products (Rao et al., 1988; Chavez et al., 2006; Benoit et al., 2015; Li et al., 2015). The hypothesis that the divergent regulatory systems affect the variations in the enzymes produced can be supported by the taxonomic distance between *P. subrubescens* and *A. niger*.

#### **4.2.4. Enzymatic saccharification of plant biomass feedstock**

The *P. subrubescens* and *A. niger* culture liquids were further used to saccharify the same combination of substrates, i.e. WB, SBP, and their equal mixture. The hydrolysis endproducts (released monosaccharides) were analysed, using HPAEC. In general, the crude enzymes mixtures from 3-day-old cultivations released higher amounts of monomeric sugars than those from 6-day-old cultivations.

##### **4.2.4.1. Saccharification of wheat bran (WB)**

The main hydrolysis product of WB was Glc (ranging from 0.73 to 3.28 mM) in all cultivation media of *P. subrubescens* and *A. niger*. The similar levels of monosaccharides released by both *P. subrubescens* and *A. niger* indicated that WB hydrolysis by cultivation supernatants was not affected by the

dissimilarities in their enzyme activity distribution. Higher amounts of Glc (1.5 fold) were released when WB was saccharified by the crude enzyme cocktail from SBP-amended cultivation of *A. niger* (2.84 mM) than with *P. subrubescens* (1.88 mM). The notably lower BGL activity of *P. subrubescens* under all cultivation conditions supported this finding. SBP-supplemented culture filtrates of both *P. subrubescens* and *A. niger* released low quantities of Xyl from WB, 0.18 and 0.13 mM respectively, indicating the low content of Xyl in SBP. No Gal, Man, Rha, GluA, and GalA were detected in the hydrolysis endproducts, which corresponds to their low concentrations in WB.

#### **4.2.4.2. Saccharification of sugar beet pulp (SBP)**

The *A. niger* cultivation supernatant was more potent in releasing monosaccharides from SBP than was *P. subrubescens*, with the exception of Gal. A two-fold higher amount of Frc was released from SBP (ranging from 2.05 to 2.53 mM) than from WB (ranging from 1.01 to 1.23 mM) by both species under all cultivation conditions. The Frc released, probably by SUC activity, originated from sucrose, which is present in both SBP and WB. In addition, low amounts of Xyl were detected after the saccharification of SBP (ranging from 0.00 to 0.04 mM) which was supported by the low BXL detected in the SBP cultivations. Alpha-xylosidase (AXL), rather than BXL activity is required to release Xyl from SBP, since it was assumed that Xyl is present in SBP in the form of xyloglucan rather than xylan (Vincken et al., 1997).

In general, these results indicate that there is a well-defined association between substrate composition and the enzyme cocktail produced by the fungi examined. With regard to the current study, fungal growth on a particular lignocellulosic substrate leads to production of a mixture of enzymes that is especially suitable for hydrolysis of this particular feedstock (Baker et al., 1997; McMillan et al., 2001). Our results are also supported by previous studies, in

which proteomic analysis of the extracellular enzyme cocktails produced by the hypercellulolytic fungi *P. funiculosum* and *P. oxalicum* revealed their potential for hydrolysis of lignocellulosic substrates while growing on complex plant-derived biomass (de Castro et al., 2010; Maeda et al., 2013; Song et al., 2016). Furthermore, the entire genome sequence of *P. subrubescens* FBCC1632 supports its hydrolytic capacity (Peng et al., 2017). Data from comparative genomics analyses demonstrated that the fungus has a higher number of genes encoding plant biomass degradation-related CAZymes, particularly GHs, than other cellulolytic fungi, such as *A. niger* or *A. oryzae*. Genome analysis of *P. subrubescens* revealed a higher number of CAZyme genes than other *Penicillia*, such as *P. chrysogenum*. Interestingly, a genome draft of *P. subrubescens* revealed enriched CAZy genes responsible for inulin degradation, which fits well with its high inulinase activity (Peng et al., 2017).

#### **4.3. Heterologous production and characterization of a new feruloyl esterase, FaeC, from *Aspergillus niger* N402 (III)**

*P. subrubescens* and *A. niger* N402 were investigated for the production of FAEs, the accessory enzymes that facilitate the decomposition of plant biomass. Since no FAE activity was detected for *P. subrubescens* under the cultivation conditions examined, the production of FAE by *A. niger* was further studied in detail. Here, a new FAE, FaeC, from *A. niger* was recombinantly produced in the yeast *Pichia pastoris*, biochemically characterized and its hydrolytic activity on complex plant biomass was also investigated. Furthermore, induction of FaeC expression in various polysaccharides and phenolic compounds was further examined.

### 4.3.1. Phylogenetic analysis

Based on phylogenetic analyses, Dilokpimol et al. (2016) classified the putative FAEs into 13 subfamilies (SF1-SF13). The members of SF1-SF3 and SF5-SF7 remain classified in the same subfamilies as in the previous phylogeny-based classification (Benoit et al., 2008). Phylogenetic analysis showed that FaeC (An12g02550) belongs to SF5, whereas the previously characterized FaeA and FaeB from *A. niger* belong to SF7 and SF1, respectively. Moreover, FaeC is also classified in the CE1 family in the CAZy database (Lombard et al., 2014). The FaeC exhibited amino-acid sequence similar to those of other characterized FAEs of SF5, such as *A. nidulans* AN5267 (86%; Debeire et al., 2012), *Aspergillus clavatus* AcFAE (81%; Damasio et al., 2013), *Neurospora crassa* NcFaeD (49%; Crepin et al., 2004), and *Myceliophthora thermophila* ClFaeA1 (47%; Kühnel et al., 2012) and ClFaeA2 (44%; Kühnel et al., 2012). The enzymes characterized from SF5 have some attributes in common, such as low molecular mass and broad substrate preference.

#### **4.3.2. Biochemical properties and substrate profile of FaeC**

Recombinant FaeC (rFaeC) was produced in *P. pastoris* as a 30-kDa protein consistent with the FAEs characterized from SF5, with molecular masses of 28-36 kDa (Crepin et al., 2004; Debeire et al., 2012; Kühnel et al., 2012; Damasio et al., 2013). Based on deglycosylation studies, rFaeC was not glycosylated, which is in contrast to FaeA and FaeB of *A. niger* (Publication III, Fig. 3A). The rFaeC illustrated wide substrate specificity, similar to that FaeB. Apart from chlorogenic acid and methyl hydroxybenzoate, it hydrolysed all other hydroxycinnamates used as substrates (Publication III, Table 1). The rFaeC showed highest activity at neutral pH of 7.0 (Publication III, Fig. 3B), similar to AcFAE and ClFaeA1 (Kühnel et al., 2012; Damasio et al., 2013). It maintained high levels of activity at pH ranges from 5.0 to 7.0. FaeA and FaeB showed highest activity at pH values of 5.0-6.0 (Faulds & Williamson, 1994; de Vries et al., 1997, 2002). The rFaeC was most active at 50°C (Publication III, Fig. 3D), which is higher than previously described for AcFAE (30°C) and ClFaeA1 (45°C) (Kühnel et al., 2012; Damasio et al., 2013), but similar to the optimum temperature of FaeB (50°C) (Levasseur et al., 2004). Nevertheless, FaeA is most active at slightly higher temperatures (55-60°C) (Faulds & Williamson, 1994; de Vries et al., 1997).

#### **4.3.3. Hydrolysis of feruloylated polysaccharides by recombinant FaeC**

To evaluate the hydrolytic efficiency of rFaeC, four insoluble feruloylated polysaccharides (wheat arabinoxylan, WB, sugar beet pectin and NaOH-solubilized wheat arabinoxylan) were used as the substrates. They were incubated with and without supplementary enzymes, including commercial xylanase and pectinases (endo-polygalacturonase (PGA), rhamnogalacturonan hydrolase and rhamnogalacturonan acetyl esterase (RGAE)). Supplementary

enzymes were used to investigate the synergistic effect of the enzymes, in addition to the substrate pretreatments. The rFaeC efficiently hydrolysed both solubilized and non-solubilized wheat arabinoxylan (3 mg enzyme/g substrate), leading to release of a notable amount of ferulic acid (FA). Release of FA increased up to 6-fold when wheat arabinoxylan was pre-treated with xylanase, while pretreated WB showed no significant effect on the release of FA (Publication III, Fig. 4). However, coincubation of rFaeC with commercial xylanase, increased FA release from both wheat arabinoxylan (4-fold) and WB (2-fold).

In all samples, negligible amounts of *p*-coumaric acid and no diferulic acid were detected. Furthermore, no synergistic activity of rFaeC and selected pectinases (PGA, rhamnogalacturonan hydrolase and RGAE) was observed. Therefore, these pectinases seemingly cannot alter the particular part of pectin that FaeC acts upon. Accordingly, debranching enzymes such as endo-arabinanase (ABN) and endo-galactanase could be used to enhance FA release from sugar beet pectin (de Vries et al., 2000). As in FaeA, FaeC favours wheat arabinoxylan, while FaeB hydrolyses SBP more efficiently (Kroon et al., 1999; de Vries et al., 2002).

#### **4.3.4. Expression of *faeC* in the presence of feruloylated polysaccharides**

*A. niger* N402 was cultivated on the ferulic acid-containing plant biomasses, WB and SBP, with or without calcium lignin over a time course of 3 days to evaluate the expression level of the *fae* genes. The expression levels were quantified by real-time PCR. The highest expressed gene in WB was *faeA*. WB also induced low expression levels of *faeC* and *faeB*. The *faeA* is seemingly the main FAE enzyme involved in FA released from WB. Calcium lignin in the growth medium increased the expression of *faeA* in WB and *faeB* in SBP on



the first day of cultivation. Low expression levels of *faeC* were detected on day 1, but increased over time. In contrast, the expression levels of *faeA* and *faeB* were relatively high on the first day of induction, but decreased after 3 days. The time-dependent expression pattern of the *fae* genes suggests that a complex gene regulation mechanism controls the process of substrate breakdown. It is influenced by the presence of the substrate and expression of other genes encoding lignocellulose-degrading enzymes (Faulds et al., 1997; de Vries & Visser, 1999; Wong, 2006). Furthermore, high *faeA* expression on WB can influence the *faeB* and *faeC* expression levels. Since the *faeA* gene is induced by both Xyl (de Vries and Visser, 1999) and FA, the *faeA* is seemingly first induced by Xyl which results in the release of FA, subsequently inducing the expression of *faeB* and *faeC*.

#### **4.3.5. Expression of *faeC* in the presence of monomeric phenolic compounds**

The effect of phenolic compounds on the expression of *faeC* was investigated in *A. niger* cultivations amended with 0.03% monomeric phenolic compounds. The effect of most monomeric phenolic compounds on the expression of *faeC* was lower than for *faeA* and *faeB*, similar to the effect of feruloylated polysaccharides (Publication III, Fig. 6). Cinnamic acid was the best inducer of *faeC* expression. Low-level expression was detected with addition of syringic, caffeic, vanillic and ferulic acids. The highest expression of *faeB* was detected when induced by FA, followed by caffeic and *p*-coumaric acids, while 3,4-dimethyl benzyl alcohol was the best *faeA* inducer. The *faeA* expression was also induced by vanillic acid and FA, but at lower levels. These results indicate that the *fae* genes of *A. niger* are induced by a wide range of monomeric phenolic compounds, but their responses are very specific. Aromatic compounds, such as 3,4-dimethoxycinnamic and sinapic acids, did

not induce *faeC* expression under the experimental conditions, but rFaeC showed activity towards them. In contrast, a previous study demonstrated correlation between the activity of FaeA and FaeB towards monomeric aromatic substrates and induction of FaeA and FaeB by these substrates (de Vries et al., 2002). In addition, FaeC can catalyse the hydrolysis of MCA, but not chlorogenic acid (an ester of caffeic acid and quinic acid). This is in contrast to FaeB, which can hydrolyse both substrates.

## 5. Conclusions

Here, a new *Penicillium* species, *P. subrubescens* FBCC1632, was isolated, identified and its potential for producing CAZymes determined. Morphological, molecular, extrolite and phylogenetic analyses of *P. subrubescens* showed that it represents a new species within section *Lanata-Divaricata* subgenus *Aspergilloides*. *P. subrubescens* was isolated from agricultural soil used for the production of tubers of Jerusalem artichoke. It produced high levels of exo-acting inulinase, which releases Frc residues from the reducing end of the inulin chain. In addition, the potential of *P. subrubescens* for producing other plant biomass-hydrolysing enzymes was examined and compared with that of *A. niger*, which is a widely studied and industrially exploited fungal species from the same class.

The growth of *P. subrubescens* in a set of plant biomass-related carbohydrates was similar to that in *A. niger*, indicating the ability to use a broad range of substrates as carbon sources. Furthermore, the cellulolytic and hemicellulolytic enzyme activities of *P. subrubescens* were higher than those of the other *Penicillia* examined. Using complex lignocellulosic substrates as carbon sources, *P. subrubescens* demonstrated ranges and levels of enzyme activities similar to those of *A. niger*. The minor variations observed may have resulted from the various regulatory systems controlling these enzymes, which will be an interesting subject for further study. In addition, our results indicated a correlation between the composition of lignocellulosic substrates and production of extracellular enzymes by the fungi examined. Moreover, the results obtained from saccharification studies revealed the ability of *P. subrubescens* to saccharify complex lignocellulosic biomasses, in a manner similar to *A. niger*. Our results indicated that *P. subrubescens* is a promising

alternative to *A. niger* in applications concerning plant-derived biomass degradation.

Efficient degradation of plant biomass requires the synergistic activity of plant biomass-acting enzymes, including (hemi)cellulases and accessory enzymes such as FAEs. A new FAE of *A. niger*, FaeC, was cloned, heterologously produced and biochemically characterized. Hydrolysis studies showed that rFaeC resulted in the release of ferulic and *p*-coumaric acids indicating active involvement of FaeC in the efficient depolymerization of complex plant-derived biomass. Moreover, FaeC and xylanase synergistically released FA from xylan. Furthermore, the differential expression of the *faeA*, *faeB* and *faeC* genes of *A. niger* in the various plant-derived substrates and monomeric phenolic compounds may point to the cooperative activity of the corresponding isoenzymes with putatively different targets, thus promoting the efficiency of the depolymerization of diverse plant biomass.

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